

SUMMARY

INVESTIGATION OF THE ANTIVIRAL ACTIVITY OF TRITERPENOID COMPOUNDS AGAINST RETROVIRUSES

by

SEYEDALI M. ARABZADEH

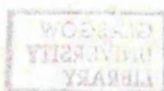
Thesis submitted for the degree of Doctor of Philosophy (PhD)

to

The Faculty of Medicine, University of Glasgow

Institute of Virology
Church Street
Glasgow G11 5JR
Scotland

March 1996



ProQuest Number: 13818414

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 13818414

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

SUMMARY

The triterpenoid compound glycyrrhizin (GL) is widely used in Japan for the treatment of viral hepatitis. GL, carbenoxolone sodium (CBX), and cicloxolone sodium (CCX) exhibit broad range antiviral activity both *in vivo* and *in vitro* (Galt *et al.*, 1992; Pompei *et al.*, 1979). Both *in vitro* and *in vivo* anti-HIV activity has also been reported for GL (Hatori *et al.*, 1989; Mori *et al.*, 1989, and 1990; Ito *et al.*, 1986 and 1988).

This thesis investigates the effect of triterpenoid compounds on replication of two retrovirus in tissue cultures; Feline leukaemia virus type A (FeLV-A) and Feline immunodeficiency virus (FIV). Both of these viruses cause AIDS-like immunodeficiency diseases in cats. The anti-retrovirus activity of the triterpenoid compounds is contrasted with the effect of the drugs on the replication of bovine herpesvirus type 1 (BHV-1) in MDBK cells.

Triterpenoid compounds are lipophilic and effect the host cell membrane functions. In order to determine drug concentrations in which cytotoxic effect of the drug could be uncoupled from the antiviral activity, cytotoxicity tests were performed. The effect of the drugs on cell culture growth and percentage cell viability were determined by trypan blue dye exclusion. The highest drug concentrations tolerated were; for FeA and CrFK cells 100 μ M CCX, or 100 μ M CBX and for MDBK cells 300 μ M CCX (used for BHV-1 studies). Drug removal experiments have shown that the effect of the drugs was reversible and that two days treatment with 100 μ M CCX, or 100 μ M CBX had no effect on the subsequent growth of FeA or CrFK cell cultures. The IC₅₀ concentrations (concentration of the drug inhibiting cellular growth by 50%) determined for both CrFK

and FeA cells after 24h or 48h drug treatments were $> 300\mu\text{M}$ and $150\mu\text{M}$ respectively. Based on the data obtained from cytotoxicity tests, it was concluded that CrFK and FeA cells could be placed within the grouping of CCX-resistant cell lines classified by Galt et al., (1990).

In order, to investigate the intracellular location of CCX molecules, an attempt was made to generate an anti-CCX antibody in immunise rabbits. Despite detection of an apparent anti-CCX antibody in an ELISA test, experiments designed to demonstrate the specific antibody tagging of CCX molecules in drug treated cells by immunofluorescence were unsuccessful.

Previous studies have shown that CCX treatment impaired the glycosylation of HSV, VSV, and SFV glycoproteins (Dargan and Subak-Sharpe 1986 and 1986a; Galt et al., 1990), suggesting that the Golgi apparatus might be a target for triterpenoid compounds. A trans-Golgi specific monoclonal antibody (directed against the p58 protein) was employed to investigate the effect of CCX on the MDBK and CrFK cells Golgi apparatus. While perinuclear (Golgi) immunofluorescence was observed in drug-free cultures, little or no such staining was detected in cells treated with CCX, suggesting that the drug perturb^s the Golgi apparatus.

It was further postulated that up-regulation of $\text{Na}^+/\text{K}^+\text{ATPase}$ activity (a known effect of triterpenoid compounds) might play a role in the effect of CCX on the Golgi apparatus. To test this hypothesis, ouabain (a specific inhibitor of $\text{Na}^+/\text{K}^+\text{ATPase}$ activity) was used in Golgi immunofluorescence labelling experiments. The effect of CCX on the Golgi apparatus of MDBK cells was impaired when cells were pre-treated

with ouabain, confirming that the CCX-induced perturbation of the trans-Golgi compartment was associated with the up-regulation of Na^+/K^+ ATPase activity. A model that can explain the effect of the triterpenoid compounds on Golgi apparatus has been given.

CBX and GL exhibit mild virucidal activity against FeLV-A, FIV, and BHV-1. 300 μM CCX treatment of virus particles in suspensions resulted in a 4 fold reduction in FeLV-A, and 10 fold reduction of FIV infectivity. Treatment of BHV-1 particles with 1500 μM GL resulted in a 4.2 fold reduction infectivity.

FeLV-A infectious virus yield, drug-dose-response experiments in which infected FeA cells were treated with increasing concentrations of CCX, CBX, or GL resulted in extracellular reductions of 155, 101, and 128 fold, when treated with 150 μM CCX, 150 μM CBX, or 2500 μM GL respectively. The kinetics of the dose-response curves obtained for each drug were triphasic; an initial ~ 10 fold reduction in infectivity when cells were treated with 25 μM CCX, 25 μM CBX, or 500 μM GL, followed by a near plateau in the curve up to 100 μM CCX, 100 μM CBX or 2000 μM GL, and a sharp decrease in the curve at 150 μM CCX, 150 μM CBX, or 2500 μM GL. The results suggest that the antiviral activity of triterpenoid compounds may operate by inhibiting two different target functions; the first, a function that enhances but is not essential for virus infectivity and which is sensitive to low drug concentrations, and the second, a function which is probably essential for virus replication and which is only sensitive to high concentrations of the drugs. However, at high drug concentrations the window between cytotoxicity and antiviral activity is very narrow. Fractionation of FeLV-A drug-dose-

response samples into cell released (CR) and cell associated (CA) virus components, revealed that CR infectivity generally decreased more rapidly than infectivity in the CA component. This finding cannot be accounted^{For} by virucidal activity alone, suggesting that the drugs might impair the release of FeLV-A particles from infected FeA cells. CCX treatment of FeLV-A infected FeA cells appeared to have little or no effect on the synthesis of virus proteins. However, investigations of the polypeptide composition of the virus particles produced in the presence of CCX revealed that the surface glycoprotein (gp70) was present in slightly reduced amounts. Whether, or not, this affected the infectivity of progeny virions is not known.

The fact that infectious FIV yields are generally low (10^3 to 10^4 p.f.u./ml) has precluded high MOI drug-dose-response experiments. Nevertheless, the potency of the anti-FIV activity of triterpenoid compounds was estimated by determination of ED50 concentrations (75 μ M CCX, and 100 μ M CBX).

SDS PAGE analysis of the protein extract from FIV-infected CrFK cells grown in the presence of 100 μ M CCX revealed that cleavage of the precursor gag p55 protein into its products p24 and p17 was diminished, suggesting impairment of post-release maturation of FIV particles. However, it is not known, whether the impairment of p55 cleavage was due to CCX molecules embedded in the plasma membrane of the infected cells, in the virus envelope, or the result of some action of the drug on the viral protease activity. ^{ed} 35 S-methionine/cysteine labelling experiments reveal_A infected cell proteins of 160K, 120K, and 41K molecular weights, these are thought to represent the FIV precursor gp160 which is cleaved to yield the gp120 surface and gp41

transmembrane proteins. The gp 160 precursor protein is not normally detected in FIV infected cell extracts and this suggest impairment, though not abolition, of the cleavage. ^{14}C -glucosamine labelled gp120 was not detected in the protein extract from FIV infected cell cultures grown in the presence of $100\mu\text{M}$ CCX, although a band of 120K was present in extracts from parallel cultures labelled with ^{35}S -methionine/cysteine. If these proteins are the same, this suggests that glycosylation of the gp120 was inhibited. The results can be interpreted as suggesting that CCX-treatment impairs transport of glycoproteins through the Golgi to the cell plasma membrane, and consequently limits the number of virus particles that can be assembled. In addition, presence of CCX molecules at the site of virus budding might impair the release of progeny virions from the cells, and presence of CCX in the envelope might impair the maturation of the virions.

Treatment of BHV-1-infected MDBK cells with $300\mu\text{M}$ CCX resulted in 4.73×10^5 and 6.95×10^4 fold reductions for CR and CA fractions respectively. Infectivity in the CR fraction decreased at a faster rate than the CA infectivity, however, virucidal activity could only account for a ~ 4 fold decrease in infectivity suggesting that other factors are involved. Electron microscopy investigation of control and drug-treated BHV-1-infected MDBK cell cultures indicated that CCX impaired assembly, maturation and release of BHV-1 virus particles, however, CCX did not impair the percent^{age} of BHV-1 particles that was enveloped. Treatment with CCX also resulted in a reduction of protein synthesis per se in MI and BHV-1-infected MDBK cells. Thus, inhibition of protein synthesis must, in part, play a role in the anti-BHV-1 activity of CCX and may

account for some of the reduction in the number of virus particles assembled. SDS PAGE investigation of BHV-1 infected cell extracts showed that BHV-1 protein synthesis was impaired although not all proteins were affected to the same extent.

The antiviral mechanism of the drugs has been described in relation to the model proposed for the effect of the drugs on the Golgi apparatus.

The work presented in this thesis is the original work of the author.

SEYEDALY M. ALI ZADAH

DECLARATION

Finally, I am indebted to my supervisor, Dr. Patrick Dargatzis, for his continued and stimulating, intelligent and enthusiastic supervision throughout this project. I would also like to thank him for his critical assessment and extensive reading of this dissertation.

The work presented in this thesis is the original work of the author.

SEYEDALI.M. ARABZADEH

ACKNOWLEDGEMENTS

Firstly, I am indebted to my supervisor Dr Derrick Dargan, for his continual and unending encouragement and enthusiastic supervision throughout this project. I would also like to thank him for his critical assessment and extensive reading of this manuscript

I would like to thank Professor J.H. Subak-Sharpe for provision of the facilities within the institute and for the overall supervision and interest in this work. I am grateful to Miss L. Baxendale and Dr. P. Thornton of the Biorex laboratories limited company (London, UK) who have supplied me with the carbenoxolone sodium and cicloxolone sodium used in this study. I would also like to thank my second supervisor Dr H. Marsden for his helpful advises, and critical reading of this manuscript. I would like to thank Mr J. Aitken for his invaluable assistance with EM studies and photography. I would like to thank the following people in the Glasgow school of veterinary medicine; Professor O. Jarrett for providing the stock of the retroviruses, cell lines, and FIV and FeLV-A antibodies used in this study, Dr Robert Osborne for his help in training me for handling FIV, and M. Golder for handling of FeLV-A; Dr M. Hosie for interesting discussions during the course of the project.

I must thank my wife and daughter in putting up with me for my continual moaning and groaning

I dedicate this thesis to my parents who supported me both emotionally and financially, without their support this work was not possible.

TABLE OF CONTENTS

	page
Summary	75
Declaration	76
Acknowledgements	77
Table of Contents	78
List of Figures	79
List of Tables	80
List of Abbreviations	80
 Chapter 1. INTRODUCTION.	 1
1.1 Antiviral compounds	2
1.1.1 Anti-herpes compounds.	2
1.1.2. Anti-influenza compounds.	9
1.1.3. Antisense antiviral agents	12
1.1.4.. Protein-protein interaction as antiviral mechanism.	13
1.1.5. Anti-HIV compounds	15
1.1.5.1. Anti-HIV compounds derived from natural sources.	26
1.1.6. Compounds with broad range antiviral activity.	29
1.1.7. Triterpenoids.	49
1.2. Retroviruses.	60
1.2.1.. Feline immunodeficiency virus (FIV).	63
1.2.2.. Feline Leukaemia virus (FeLV).	68
1.3. Bovine herpesvirus type 1 (BHV-1).	69
1.4 Purpose and aim of the project.	70
 Chapter 2. MATERIAL AND METHODS.	 70
2.1. Materials.	70
2.1.1. Chemicals	70
2.1.2. Fine Chemicals	71
2.1.3. Antibodies.	72
2.1.4. Radiochemicals.	72
2.1.5. Media.	73
2.1.6. Cell cultures.	74
2.2. Methods.	74
2.2.1. Cell culture passage.	74
2.2.2. Storage and recovery of frozen cell cultures.	75
2.2.3. Preparation of cicloxolone, carbenoxolone, glycyrrhizin, monensin, and tunicamycin solutions.	75

2.2.4.	The effect of CCX, CBX, or GL on FeA, QN10S , and CrFK cells in culture.	76
2.2.4.1.	Drug cytotoxicity tests.	76
2.2.4.2.	Reversal of the effect of CCX treatment on drug treated cultures.	77
2.2.5.	Virus cultures.	78
2.2.5.1.	Viruses.	78
2.2.5.2.	Preparation of virus stocks.	78
2.2.5.2.1.	BHV-1	78
2.2.5.2.2.	FIV and FeLV-A.	79
2.2.5.3.	FeLV-A titration.	79
2.2.5.4.	Titration and immunostaining of FIV plaques on CrFK cell monolayers.	80
2.2.5.4.1.	FIV titration.	80
2.2.5.4.2.	Immunostaining.	80
2.2.5.5.	BHV-1 titration.	81
2.2.5.6.	Virus purification.	82
2.2.5.6.1.	Virus concentration.	82
2.2.6.	Single-cycle replication of FeLV-A in resting or non-resting FeA cell cultures.	83
2.2.6.1.	Non-resting cell cultures.	83
2.2.6.2.	Resting cell cultures.	83
2.2.7.	Feline immunodeficiency virus plaque reduction assay.	84
2.2.8.	Drug infectious virus yield dose-response experiment.	
2.2.8.1.	FeLV-A.	84
2.2.8.2.	BHV-1.	85
2.2.9.	Investigation of the antiviral mechanism of CCX, CBX, and GL.	
2.2.9.1.	Virucidal activity.	86
2.2.9.2.	Polyacrylamide gel electrophoresis of polypeptides (SDS PAGE).	86
2.2.9.3.	Fixing, staining, and destaining of SDS PAGE gels.	87
2.2.9.4.	Silver-staining of proteins in SDS PAGE gels.	89
2.2.9.5.	Western immunoblotting.	89
2.2.9.6.	Immunoprecipitation.	90
2.2.9.6.1.	Preparation of protein extract for immunoprecipitation.	90
2.2.9.6.2.	Preparation of protein A sepharose beads.	91
2.2.9.6.3.	Immunoprecipitation reaction.	91
2.2.9.7.	Radiolabelling of virus and cellular proteins.	92
2.2.9.7.1.	¹⁴ C-glucosamine labelling of Mock-infected and FIV specific polypeptides and pelleting of the virus particles made in the presence of CCX.	92
2.2.9.7.2.	³⁵ S-methionine/cysteine labelling of Mock-infected and FeLV-A or FIV infected cell polypeptides and pelleting of the particles made in the presence of CCX.	93
2.2.9.7.3.	³⁵ S-methionine and ³² P-inorganic phosphate labelling of mock- infected and BHV-1 infected MDBK cell polypeptides.	94
2.2.10.	Electron microscopy.	95
2.2.10.1.	Negative staining.	95
2.2.10.2.	Epoxy-resin (EPON 812) embedding and thin sectioning of culture samples.	96

2.2.11.	Precleaning of anti-mouse FITC conjugate.	97
2.2.12.	Detection of Golgi apparatus in CCX-treated cells by immunofluorescence using anti-Golgi antibodies as markers.	97
2.2.13.	Detection of acidic compartments in CCX-treated cells by immunofluorescence using the DAMP, anti-DAMP system.	98
2.2.14.	Investigation of the effect of ouabain in combination with CCX on p58 Golgi staining of MDBK cells.	99
2.2.15.	Preparation of a rabbit antibody directed against CCX.	100
2.2.15.1.	Preparation of CCX-BSA, and CCX-KLH conjugate.	100
2.2.15.2.	Estimation of the CCX in the CCX/BSA/EDC complex.	101
2.2.15.3.	Injection of rabbits with CCX/BSA conjugate and collection of the sera	101
2.2.15.4.	Coupling of the CNBr-activated sepharose beads with bovine serum albumin (BSA).	102
2.2.15.5.	Removal of the anti-BSA antibody by passage of the sera through an anti-BSA antibody binding column.	103
2.2.15.6.	Detection of anti-CCX antibody in the rabbit sera by ELISA.	104
2.3.	Solutions.	106
Chapter 3. RESULTS		
3.1.	Investigation of the effect of triterpenoid compounds on uninfected cells in culture.	114
3.1.1.	The effect of CCX or CBX treatment on the growth and percentage viability of CrFK cells in culture.	115
3.1.2.	The effect of CCX, CBX, or GL treatments on the growth and percentage viability of QN10S cells in culture.	118
3.1.3.	The effect of CCX, or CBX treatments on the growth and percentage viability of FeA cells in culture.	122
3.1.4.	The effect of 24h CCX treatment on the viabilities of Madin-Darby Bovine Kidney (MDBK), Bovine embryo lung (BEL), and Bovine Embryo Kidney (BEK) cells in culture.	124
3.1.5.	Recovery of CCX-treated FeA and CrFK cells in culture after drug removal	125
3.1.5.1.	Reversibility of the effect of CCX on FeA cells in culture.	125
3.1.5.2.	Reversibility of the effect of CCX on CrFK cells in culture.	127
3.1.6.	Preparation of an anti-CCX antibody generated in rabbits and its detection by ELISA tests	128
3.1.7.	Investigation of the effect of CCX on MDBK and CrFK cells using Golgi-specific anti-p58 antibody.	130
3.1.8.	Investigation of the effect of ouabain in combination with CCX on the p58 Golgi staining of MDBK cells.	132
3.1.9.	Detection of the acidic compartments of the MDBK cells treated with CCX, using DAMP/anti-DAMP system.	133
3.2.	Investigation of the antiviral effect of triterpenoid compounds on the replication of Feline Leukaemia virus type A (FeLV-A).	134

3.2.1.	One step growth curve of FeLV-A in resting and non-resting FeA cells in culture.	134
3.2.2.	FeLV-A infectious virus yield/CCX, CBX, and GL dose- response experiments.	136
3.2.3.	Estimation of the virucidal activity of CCX, CBX, and GL against FeLV-A.	140
3.2.4.	Purification of FeLV-A by Ficoll gradient centrifugation, and investigation of the SDS PAGE polypeptide profile of purified virus.	141
3.2..5.	SDS PAGE polypeptide analysis of FeLV-A infected FeA cell extracts.	143
3.2.5.1.	SDS PAGE analysis of radiolabelled proteins in progeny virus particles, and in the cell extracts of FeLV-A persistently infected cell cultures treated with CCX.	145
3.3.	Investigation of the antiviral effect of triterpenoid compounds on the replication of Feline immunodeficiency virus (FIV).	147
3.3.1.	The effect of CCX and CBX treatments on the plaquing efficiency of FIV on CrFK cell monolayers.	147
3.3.2.	Estimation of the virucidal activity of CCX, CBX, or GL against FIV.	149
3.3.3.	Purification of FIV by Ficoll gradient centrifugation and investigation of the SDS PAGE polypeptide profile of purified virus.	150
3.3.4.	SDS PAGE investigation of progeny FIV virus particle proteins and protein extracts from FIV persistently infected CrFK cell cultures treated with CCX.	152
3.4.	Investigation of the effect of the triterpenoid compounds on the replication of Bovine herpesvirus type 1 (BHV-1).	162
3.4.1.	The effect of CCX treatment on the 24h infectious BHV-1 yield from MDBK cells in culture.	162
3.4.2.	Investigation of the virucidal activity of CCX, CBX, and GL against BHV-1.	163
3.4.3.	Thin section transmission electron microscope studies of BHV-1 infected and MI MDBK cell cultures treated with increasing concentrations of CCX.	164
3.4.4.	SDS PAGE investigation of the MI and BHV-1 infected cell proteins with ³⁵ S-methionine and ³² P-orthophosphate .	166

Chapter 4. DISCUSSION

4.1	Cytotoxicity.	171
4.2.	Anti-CCX antibody.	172
4.3.	The effect of CCX on the Golgi apparatus.	174
4.4.	A model to explain the effect of CCX on Golgi apparatus.	175
4.5.	Other cellular mechanisms which might be affected by treatment with triterpenoid compounds.	179
4.6.	Antiviral activity against FeLV-A.	181

LIST OF FIGURES

4.6.1.	Proposed antiviral mechanism against FeLV-A	182
4.7.	Antiviral activity against FIV.	183
4.7.1.	Proposed antiviral mechanism against FIV.	184
4.8.	Antiviral activity against BHV-1.	185
4.8.1.	Proposed antiviral mechanism against BHV-1.	187
4.9.	Final conclusions.	189
4.10.	Future work.	191
	References	

FIGURE 1.

Structure of anti-HIV compounds.

FIGURE 4.

Structure of anti-HIV compounds derived from natural sources.

FIGURE 5.

Structure of interferon compounds.

FIGURE 6.

Schematic representation of HIV, FIV, and FeLV 3' gene organization and structure.

FIGURE 7A.

The effect of CCX on CrFK cell viability.

FIGURE 7B.

The effect of CCX on CrFK cell growth (Replication index).

FIGURE 8A.

The effect of CBX on CrFK cell viability.

FIGURE 8B.

The effect of CBX on CrFK cell growth (Replication index).

FIGURE 9A.

The effect of CCX on QN103 cell viability.

FIGURE 9B.

The effect of CCX on QN103 cell growth (Replication index).

FIGURE 10A.

The effect of CBX on QN103 cell viability.

FIGURE 10B.

The effect of CBX on QN103 cell growth (Replication index).

LIST OF FIGURES

<u>FIGURE 1.</u>	Structure of anti-herpes compounds.	After page 8
<u>FIGURE 2.</u>	Structure of amantadine, rimantadine, ribavirin, foscarnet, and PMEA.	11
<u>FIGURE 3.</u>	Structure of anti-HIV compounds.	21
<u>FIGURE 4.</u>	Structure of anti-HIV compounds derived from natural sources	25
<u>FIGURE 5.</u>	Structure of triterpenoid compounds.	39
<u>FIGURE 6.</u>	Schematic representation of HIV, FIV, and FeLV-A genomic organisation.	64
<u>FIGURE 7A.</u>	The effect of CCX on CrFK cell viability.	117
<u>FIGURE 7B.</u>	The effect of CCX on CrFK cell growth (Replication index).	117
<u>FIGURE 8A.</u>	The effect of CBX on CrFK cell viability.	117
<u>FIGURE 8B.</u>	The effect of CBX on CrFK cell growth (Replication index).	117
<u>FIGURE 9A.</u>	The effect of CCX on QN10S cell viability.	121
<u>FIGURE 9B.</u>	The effect of CCX on QN10S cell growth (Replication index).	121
<u>FIGURE 10A.</u>	The effect of CBX on QN10S cell viability.	121
<u>FIGURE 10B.</u>	The effect of CBX on QN10S cell growth (Replication index).	121

<u>FIGURE 11A.</u>	
The effect of GL on QN10S cell viability.	121
<u>FIGURE 11B.</u>	
The effect of GL on QN10S cell growth (Replication index).	121
<u>FIGURE 12A.</u>	
The effect of CCX on FeA cell viability.	123
<u>FIGURE 12B.</u>	
The effect of CCX on FeA cell growth (Replication index).	123
<u>FIGURE 13A.</u>	
The effect of CBX on FeA cell viability.	123
<u>FIGURE 13B.</u>	
The effect of CBX on FeA cell growth (replication index).	123
<u>FIGURE 14.</u>	
The effect of 24h treatment with CCX on MDBK, BEL, and BEK cell viability.	124
<u>FIGURE 15.</u>	
Immunofluorescent labelling of Golgi apparatus of CrFK cells with anti-p58 antibody.	131
<u>FIGURE 16.</u>	
Immunofluorescent labelling of Golgi apparatus of MDBK cells with anti-p58 antibody.	131
<u>FIGURE 17.</u>	
Immunofluorescent labelling of Golgi apparatus of MDBK cells with anti-p58 antibody.	132
<u>FIGURE 18.</u>	
Immunofluorescent labelling of MDBK cells with DAMP, anti-DAMP antibody.	133
<u>FIGURE 19.</u>	
One step growth of FeLV-A in resting and non-resting FeA cell cultures.	135

<u>FIGURE 20.</u>	
The effect of CCX treatment on the 24h infectious yield of FeLV-A from FeA cells.	139
<u>FIGURE 21.</u>	
The effect of CBX treatment on the 24h infectious yield of FeLV-A from FeA cells.	139
<u>FIGURE 22.</u>	
The effect of GL treatment on the 24h infectious yield of FeLV-A from FeA cells.	139
<u>FIGURE 23.</u>	
FeLV-A particles purified on a 5-15% Ficoll gradient.	141
<u>FIGURE 24.</u>	
Electron micrograph of purified Ficoll-banded FeLV-A virus, middle band.	141
<u>FIGURE 25.</u>	
Electron micrograph of purified Ficoll-banded FeLV-A virus, bottom band..	141
<u>FIGURE 26.</u>	
Silver-stained polypeptide profiles of purified FeLV-A particles obtained on a 10% SDS PAGE minigel..	141
<u>FIGURE 27.</u>	
Western blot of the polypeptide profiles obtained on a 10% SDS PAGE gel of FeLV-A infected FeA cells.	144
<u>FIGURE 28.</u>	
Polypeptide profile obtained on a 10% SDS PAGE gel of ³⁵ S-methionine/cysteine labelled proteins in the extracts from MI or FeLV-A infected FeA cells.	146
<u>FIGURE 29.</u>	
Polypeptide profiles obtained on a 10% SDS PAGE gel of ³⁵ S-methionine/ cysteine labelled proteins pelleted from the culture medium of MI or FeLV-A infected Fe A cells.	146

<u>FIGURE 30.</u>	
The effect of CCX, and CBX on plaquing efficiency of FIV.	148
<u>FIGURE 31.</u>	
FIV particles purified on a 5-15% Ficoll gradient.	151
<u>FIGURE 32.</u>	
Electron micrograph of purified FIV particles banded on Ficoll gradients.	151
<u>FIGURE 33.</u>	
Silver-stained polypeptide profile of purified FIV particles obtained on a 10% SDS PAGE gel.	151
<u>FIGURES 34 A.</u>	
Polypeptide profiles obtained on a 10% SDS PAGE gel of ³⁵ S-methionine/cysteine labelled proteins pelleted from the culture medium of MI or FIV-infected and CrFK cells.	157
<u>FIGURE 34 B.</u>	
Polypeptide profiles obtained on a 10% SDS PAGE gel of ³⁵ S-methionine/cysteine labelled proteins in the extracts from MI or FIV-infected CrFK cells.	157
<u>FIGURE 35.</u>	
Polypeptide profiles obtained on a 7.5% SDS PAGE gel of proteins pelleted from MI or FIV-infected CrFK cells. Cells were labelled with either ³⁵ S-methionine/ cysteine or ¹⁴ C-glucosamine.	158
<u>FIGURE 36.</u>	
Polypeptide profiles obtained on a 7.5% SDS PAGE gel of extracts from MI or FIV-infected CrFK cells. Cells were labelled with either ³⁵ S-methionine/cysteine or ¹⁴ C-glucosamine.	159
<u>FIGURE 37.</u>	
The effect of CCX treatment on the 24h infectious BHV-1 yield from MDBK cells.	162
<u>FIGURE 38.</u>	
The effect of CCX on incorporation of ³⁵ S-methionine or BHV-1 infected MDBK cell proteins.	169

LIST OF TABLES

FIGURE 39.

Polypeptide profile obtained on a 10% SDS PAGE gel of the ^{35}S -methionine labelled proteins in the extracts from BHV-1 infected and MI of MDBK cells.

169

FIGURE 40.

Polypeptide profiles obtained on a 9% SDS PAGE gel of MI MDBK cell extracts from cultures labelled with either ^{35}S -methionine, or ^{32}P -orthophosphate.

169

FIGURE 41.

Polypeptide profiles obtained on a 9% SDS PAGE gel of BHV-1 infected MDBK cell extracts from cultures labelled with either ^{35}S -methionine, or ^{32}P -phosphate.

169

FIGURE 42.

Model to explain the effect of CCX on the Golgi apparatus.

178

TABLE 3.

Detection of an anti-CCX antibody: ELISA controls.

179

TABLE 4.

Detection of an anti-CCX antibody: ELISA test results.

179

TABLE 5.

FoU-W-A: Virucidal activity of CCX, CBX, and GL determined after 24h incubation at 4°C (a) or 37°C (b).

180

TABLE 6.

FIV: Virucidal activity of CCX, CBX, and GL determined after 24h incubation at 4°C (a) or 37°C (b).

181

TABLE 7.

BHV-1: Virucidal activity of CCX, CBX, and GL determined after 24h incubation at 4°C (a), or 37°C (b).

182

TABLE 8.

The effect of CCX treatment on: number of empty and cored BHV-1 particles, number of enveloped particles in cytoplasm, and number of viruses in extracellular medium.

183

LIST OF TABLES

<u>TABLE 1 A.</u>	After page
The effect of CCX on FeA cell number and percentage viability before drug removal .	126
<u>TABLE 1 B.</u>	
The effect of CCX removal on FeA cell number and cell viability, after 1, 2, or 3 days of drug treatment.	126
<u>TABLE 2 A.</u>	
The effect of CCX on CrFK cell number and percentage viability before drug removal.	128
<u>Table 2 B.</u>	
The effect of CCX removal, on CrFK cell number and cell viability after 1, 2, or 3 days drug treatment.	128
<u>TABLE 3.</u>	
Detection of an anti-CCX antibody; ELISA controls.	129
<u>TABLE 4.</u>	
Detection of an anti-CCX antibody; ELISA test results.	129
<u>TABLE 5.</u>	
FeLV-A: Virucidal activity of CCX, CBX, and, GL determined after 24h incubation at 4°C (a) or 37°C (b).	140
<u>TABLE 6.</u>	
FIV: Virucidal activity of CCX, CBX, and GL determined after 24h incubation at 4°C (a) or 37°C (b).	149
<u>TABLE 7.</u>	
BHV-1: Virucidal activity of CCX, CBX, and GL determined after 24h incubation at 4°C (a), or 37°C (b).	163
<u>TABLE 8.</u>	
The effect of CCX treatment on: number of empty and cored BHV-1 particles, number of enveloped particles in cytoplasm, and number of viruses in extracellular medium.	165

TABLE 9.

The effect of CCX treatment on: percent cell sections containing virus particles, percent of empty and DNA containing particles, and percent enveloped BHV-1 particles in cytoplasm . 165

TABLE 10.

Average percent cell viability determined at 48h after CCX treatment. 172

TABLE 11.

Average CCX IC50 and RI at IC50, of sensitive and intermediate cell lines in comparison to FeA and CrFK cells. 172

ALT	Aspartate Transferase
BEX	Bovine Embryo Kidney
BEL	Bovine embryo fibroblast
BHV-1	Bovine herpesvirus
BSA	Bovine serum albumin
CBX	Carbenoxolone sodium
CCX	Cicloexolone sodium
CrFK	Crandell feline kidney
FC	Foetal calf serum
FeA	Feline embryo fibroblast
FeLV-A	Feline Leukemia virus type A
FIV	Feline immunodeficiency virus
GL	Glycyrrhizin
HIV	Human immunodeficiency virus
MDCK	Madin-Darby Bovine Kidney
MON	Monensin
PI	Preimmune
PBS	Phosphate buffer saline
SNMC	Stronger Neo-Minophagen C
TUN	Tunicamycin
ED50	Effective Dose

LIST OF ABBREVIATIONS

ALT	Alanine Transferase	Ad-5	Adenovirus type 5
AST	Aspartate Transferase		
BEK	Bovine Embryo Kidney		
BEL	Bovine Embryo Lung		
BHV-1	Bovine herpesvirus.		
BSA	Bovine serum albumin		
CBX	Carbenoxolone sodium		
CCX	Cicloxolone sodium		
CrFK	Crandell feline kidney		
Fc	Foetal calf serum		
FeA	Feline embryo fibroblast		
FeLV-A	Feline Leukemia virus type A		
FIV	Feline immunodeficiency virus		
GL	Glycyrrhizin		
HIV	Human immunodeficiency virus		
MDBK	Madin-Darby Bovine Kidney		
MON	Monensin		
PI	Preimmune		
PBS	Phosphate buffer saline	Reo-3	Reovirus type 3
SNMC	Stronger Neo-Minophagen C		
TUN	Tunicamycin	SFV	Semliki Forest virus
ED ₅₀	EFFECTIVE DOSE		

1.1. ANTIVIRAL COMPOUNDS

Two strategies have been employed to control virus infection: vaccination and antiviral chemotherapy. Vaccination for the control of smallpox virus began in 18th century, and in recent years resulted in world-wide eradication of smallpox virus. Vaccines for polio, measles, and chicken pox are the principal method for the control of infections by these viruses. Vaccines, however, may be of limited use for the control of viruses which exhibit a latent phase in their life cycle (herpesviruses and HIV), and here antiviral chemotherapy is used to control the infection. However, in contrast to development of antibacterial agents, the development of antiviral compounds faces certain obstacles, as viruses use much of the cellular machinery in their replication.

1. CHAPTER ONE

INTRODUCTION

Some viruses, such as herpesviruses have replication mechanisms which are unique to these viruses and are not found in the host cell. Some of the steps in this pathway have been successfully targeted by antiviral compounds. Unfortunately, there are few antiviral agents which offer high selectivity. In addition, most of the antiviral compounds are only effective against actively replicating viruses, and at present no antiviral compound is being used successfully in inhibition of latent viruses.

Some of the antiviral compounds, which are used chemically to treat or prevent viral diseases in antiviral chemotherapy are discussed below.

1.1.1. ANTHERPES COMPOUNDS

ACYCLOVIR, ACYVIRAL, CICLOVIR

Acyclovir [9-(3-hydroxyethylthymine) guanine] (ZoviraxTM) (Figure 1) is a

1.1. ANTIVIRAL COMPOUNDS.

Two strategies have been employed to control virus infection: vaccination and antiviral chemotherapy. Vaccination for the control of smallpox virus began in 18th century, and in recent years resulted in world-wide eradication of smallpox virus. Vaccines for polio, measles, and rubella are the principal method for the control of infections by these viruses. Vaccines, however, may be of limited use for the control of viruses which exhibit a latent phase in their life cycle (herpesviruses and HIV), and here antiviral chemotherapy is used to control the infection. However, in contrast to development of antibacterial agents, the development of antiviral compounds faces greater obstacles, as viruses use much of the cellular machinery in their replication.

Some viruses, such as herpesviruses have replication mechanisms which are unique to these viruses and are not found in the host cell. Some of the steps in this pathway have been successfully targeted by specifically designed antiviral compounds. Unfortunately, there are few antiviral agents which offer high selectivity. In addition, most of the antiviral compounds are only effective against actively replicating viruses, and at present no antiviral compound is being used successfully in inhibition of latent viruses.

Some of the antiviral compounds, which are used clinically or exhibit a promising future in antiviral chemotherapy are discussed below.

1.1.1. ANTIHERPES COMPOUNDS.

ACYCLOVIR (ACV)/VALCICLOVIR.

Acyclovir [9-(2-hydroxyethoxymethyl) guanine] (Zovirax™) (Figure 1) is a

nucleoside analogue of guanosine and has selective antiviral activity against herpesviruses. ACV is activated upon phosphorylation by the viral specific thymidine kinase to yield ACV-monophosphate. Further phosphorylation to ACV-diphosphate (ACV-DP) and ACV-triphosphate (ACV-TP) is carried out by cellular kinases (Fyfe *et al.*, 1978). ACV-TP is both a competitive inhibitor of dGTP and a substrate for viral DNA polymerase. ACV-TP becomes incorporated into the growing viral DNA chain but, because ACV-triphosphate lacks the 3' hydroxyl group, the viral DNA polymerase can not add the next dNTP and the whole process is terminated (Furman *et al.*, 1984).

The antiviral activity of ACV-triphosphate is selective for herpes-infected cells and, although a small amount of ACV-TP has been found in extracts from uninfected Vero cells, the rate of conversion of ACV to ACV-TP is 30-120 times faster in HSV-1-infected Vero cells (Fyfe *et al.*, 1978). ACV is not effective against those herpesviruses which lack a thymidine kinase gene (TK) (e.g. CMV). ACV-resistance can be conferred by mutations in two different loci- the sequences encoding the TK gene or those encoding the DNA POL gene (Burns *et al.*, 1982; Coen and Schaffer, 1980).

Acyclovir is widely used both topically and systemically for the treatment of herpes simplex virus infections. The ED₅₀ value for HSV can be as low as 0.1 μ M (Collins *et al.*, 1983) but, for HCMV, the ED₅₀ can be as high as 200 μ M (Balfour *et al.*, 1988). VZV and EBV exhibit intermediate sensitivity to ACV with ED₅₀'s (between 1.3 to 20.6 μ M and 0.3 to 25 μ M respectively) depending upon the viral strain and the cell line used (Balfour *et al.*, 1988; Biron and Elion, 1980). Due to the poor oral bioavailability of ACV (about 20%) (De Miranda and Blum, 1993), much higher oral or intravenous doses of ACV have been

administered to obtain clinically beneficial results against infections caused by less sensitive herpesviruses (e.g. EBV)

Several prodrug derivatives of acyclovir have been investigated to identify compounds with increased oral bioavailability. One such drug is valciclovir (the L-valyl ester of acyclovir), the oral bioavailability of which is about 3-5 times higher than that of oral acyclovir (Blum *et al.*, 1994). The antiviral activity of valciclovir is entirely due to its rapid and complete conversion to acyclovir *in vivo* (Beutner, 1995).

PENCICLOVIR/FAMCICLOVIR.

Penciclovir [9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine] (Figure 1) is also ^{antiviral} derivative of guanosine and consequently has a similar (but not identical) mode of action (Earnshaw *et al.*, 1992). Penciclovir is active against HSV, VZV, and EBV (Boyd *et al.*, 1987 and 1988; Bacon *et al.*, 1995). Penciclovir-TP is 10 to 20 times more stable than ACV-TP (Earnshaw *et al.*, 1992) thus, PCV, like valciclovir might be administered less frequently than ACV in treatment of herpesvirus infections. Indeed famciclovir (the prodrug of penciclovir) has already been licensed for use in patients suffering from herpesvirus infections (Carrington, 1994). Famciclovir is converted to penciclovir giving an absolute oral bioavailability of about 77% which is better than that reported following the conversion for acyclovir following conversion from valaciclovir (Pue *et al.*, 1993)

GANCICLOVIR.

Ganciclovir {[9-(1,3-dihydroxy-2-propoxymethyl-guanine)]; DHPG, (Figure 1) like ACV, is selectively phosphorylated to ganciclovir-monophosphate by HSV-TK. Ganciclovir-TP is a potent inhibitor of HSV DNA polymerase. Oral ganciclovir (GCV) treatment is more than 50 fold ^{more} effective than oral ACV in the treatment of systemic or local HSV-1 or HSV-2 intravaginal infection in mice (Smee *et al.*, 1983). This may be due to the fact that GCV is more stable *in vivo* than ACV, however, prolonged ganciclovir therapy is associated with bone marrow suppression resulting in severe neutropenia (Fan-Havard *et al.*, 1989). That is why GCV is not used in therapeutics against HSV-infections in humans. Ganciclovir, however, is important as a potent inhibitor of human cytomegalovirus (HCMV). The mechanism of GCV anti-HCMV activity is essentially the same as its anti-HSV activity (Mar *et al.*, 1985). However HCMV does not code a virus-specific thymidine kinase but, the HCMV UL97 gene product has been found to be responsible for the phosphorylation of GCV in HCMV infected cells (Sullivan *et al.*, 1992 and Littler *et al.*, 1992). Ganciclovir has marginal oral bioavailability in man and is administered systemically. GCV is employed for the treatment of HCMV-induced retinitis, particularly in immunocompromised patients (AIDS patients or allograft recipient on immunosuppressive drug transplant and are receiving immunosuppressive drugs) (Fan-Havard *et al.*, 1989). GCV-resistant mutants (usually mutated in the UL97 gene) have been isolated from patients. (Stanat *et al.*, 1991; Sullivan *et al.*, 1992). However, GCV-resistant virus with mutations in the POL gene have been isolated from the AD169 lab strain of HCMV. (Sullivan *et al.*, 1993).

HPMPC (CIDOFOVIR)

HPMPC belongs to the group of phosphonylmethoxyalkyl derivative of purines and pyrimidines (Figure 1). These compounds have broad-range antiviral activity against DNA ^{and retroviruses} viruses (DeClercq *et al.*, 1986). (S)-HPMPC is the most active of these compounds with an ED₅₀ of about 0.1 µg/ml for HCMV and a therapeutic index of 1500 (Snoek *et al.*, 1988). HPMPC is converted to HPMPC-diphosphate (the active form of the compound) by cellular enzymes in both infected and uninfected cells (Bronson *et al.*, 1990). HPMPC-DP is a competitive inhibitor of viral DNA polymerase and has a higher affinity for HCMV DNA POL than for cellular DNA polymerase (Ho *et al.*, 1992). HPMPC-DP has a half life in excess of 48h with activity against GCV-resistant and GCV-sensitive HCMV (Bronson *et al.*, 1991; Stanat *et al.*, 1991). In a study involving 21 asymptomatic HIV-1 infected patients receiving HPMPC intravenously (0.5 or 1.5mg/kg body weight twice weekly), HCMV was cleared from the urine. None of the patients in the study had adverse side-effects resulting from drug treatment. However, patients receiving 5mg/Kg body weight twice weekly developed renal dysfunction (Polis *et al.*, 1995).

IDOXURIDINE (IDU).

IDU was the first antiviral agent to be licensed for use in the treatment of HSV-induced keratitis (Figure 1). The drug was applied topically but proved too toxic for systemic use.

Cellular or virus specific thymidine kinase enzymes are capable of phosphorylating IDU to IDU-monophosphate (Littler *et al.*, 1986) however, the affinity of IDU for virus


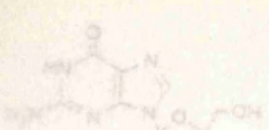
TK is slightly better than that ^{for} the cellular TK. Therefore, phosphorylation of IDU is more efficient in virus-infected cells, conferring a small measure of antiviral selectivity. HSV TK⁻ mutants are less susceptible to IDU than the wild type virus (Field *et al.*, 1981). Following phosphorylation *in vivo* and incorporation into DNA molecules, IDU inhibits both viral and cellular synthesis. However, IDU also has been reported to inhibit several cellular and viral regulatory enzymes (thymidine kinase, thymidylate kinase, and DNA polymerase) (Prusoff and Lin, 1988).

5-TRIFLUOROMETHYL-2'-DEOXYURIDINE (TFT) (TRIFLURIDINE).

TFT has also been used topically for the treatment of HSV keratitis (Figure 1). Like IDU, TFT is too toxic for systemic use. Cellular enzymes are largely responsible for conversion of TFT to TFT-TP. TFT-TP is a substrate for, and inhibitor of both viral and cellular DNA polymerases (Prusoff *et al.*, 1984).

ADENINE ARABINOSIDE (Ara-A) (VIDARABINE).

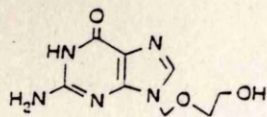
Adenine arabinoside was the first compound licensed for systemic use in the treatment of herpesvirus infections (Whitely *et al.*, 1977) (Figure 1). *In vivo*, Ara-A is deaminated by cellular enzyme adenosine deaminase to 9- β -D arabinofuranosylhypoxanthine (AraHx) which has less activity than Ara A. Inhibitors of adenosine deaminase [such as erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA)] can therefore, increase the antiviral activity of Ara-A *in vivo*. Ara-A is poorly soluble in water and therefore needs to be administered intravenously with a large volume of liquid.



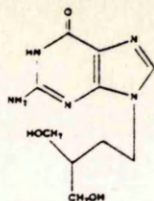
Cellular adenosine and deoxycytidine kinase converts Ara-A to Ara-A-monophosphate (Ara-A-MP) which is more soluble than Ara-A and is not deaminated by adenosine deaminase. Ara-A-MP is further converted to Ara-A-TP (the active form) by cellular enzymes. Ara-A-TP competes with dATP as substrate for both cellular and viral polymerases. Thus, Ara-A-TP inhibits viral DNA polymerase and/or becomes incorporated into the growing DNA chain, making DNA elongation less likely to occur (Reid *et al.*, 1988). HSV-DNA polymerase, however, is 15 fold more sensitive to Ara-A-TP inhibition than cellular DNA polymerase (Ostrander and Cheng, 1980).

Ara-A has been shown to be effective against HSV-1, HSV-2, (including TK⁻ HSV mutants), VZV, and HCMV. Ara-A has been used mainly for the treatment of HSV-encephalitis. Ara-A-resistant HSV mutants with mutation in the POL gene have been isolated both *in vitro* and *in vivo* (Coen *et al.*, 1982).

FIGURE 1. Mechanism of action of Ara-A.



ACV

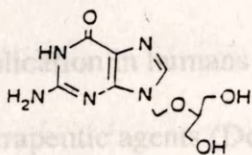


PCV

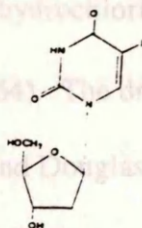
1.1.2. ANTI-INFLUENZA AGENTS

AMANTADINE AND RIMANTADINE

Amantadine (1-amino-7-adamantane hydrochloride) and its derivative rimantadine (α -methyl-1-adamantane methylamine hydrochloride) (Figure 2) inhibit influenza A virus replication in humans (Davis *et al.*, 1964). These drugs are effective both as prophylactic and therapeutic agents (Dolin *et al.*, 1982 and Douglas *et al.*, 1993).

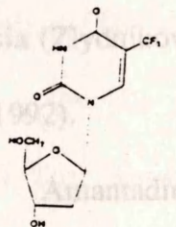


GCV

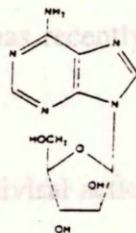


IDU

Although the US FDA approved the use of oral amantadine in the prevention and treatment of influenza virus infections in 1966, the use of these drugs in the developed world has generally been very limited. Rimantadine, however, has been used extensively in Russia (Tikhonov *et al.*, 1981), and has recently been licensed for use in France (May *et al.*, 1992).

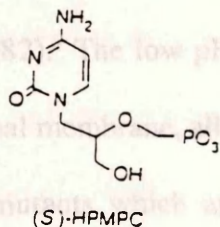


TFT



Ara-A

Amantadine exerts different antiviral actions depending on the concentration of the drug used. At high concentrations ($>0.1 \mu\text{M}$), influenza virus infection of cells is inhibited by elevation of endosomal pH (Daniels *et al.*, 1985). Like many enveloped RNA viruses, influenza virus enters cells by receptor mediated endocytosis (Wallace *et al.*, 1986 and Helms *et al.*, 1987). The low pH of the endosomes prompts fusion of the viral envelope with the endosomal membrane, allowing release of the virus core and initiation of infection. Influenza virus mutants, which are resistant to high concentrations of amantadine, have hemagglutinin (HA) proteins which fuse with cellular membranes at a higher pH than those of the wild type virus (Daniels *et al.*, 1985). The replication of a number of enveloped RNA viruses, dependent upon receptor mediated endocytosis, are inhibited by amantadine at



(S)-HPMPC

FIGURE 1. Structure of anti-herpes compounds.

1.1.2. ANTI-INFLUENZA AGENTS.

AMANTADINE AND RIMANTADINE.

Amantadine (1-aminoadamantane hydrochloride) and its derivative rimantadine (α -methyl-1-admantane methylamine hydrochloride) (Figure 2) inhibit influenza A virus replication in humans (Davis *et al.*, 1964). The drugs are effective both as prophylactic and therapeutic agents (Dolin *et al.*, 1982 and Douglas *et al.*, 1990).

Although the US FDA approved the use of oral amantadine in the prevention and treatment of influenza virus infections in 1966, the use of these drugs in the developed world has generally been very limited. Rimantadine, however, has been used extensively in Russia (Zlydnikov *et al.*, 1981), and has recently been licensed for use in France (Hay *et al.*, 1992).

Amantadine exerts different antiviral actions depending on the concentration of the drug used. At high concentrations ($> 0.1\text{mM}$), influenza virus infection of cells is inhibited by elevation of endosomal pH (Daniels *et al.*, 1985). Like many enveloped RNA viruses, influenza virus enters cells by receptor mediated endocytosis (Wallbank *et al.*, 1966 and Helinus *et al.*, 1982). The low pH of the endosomes prompts fusion of the viral envelope with the endosomal membrane, allowing release of the virus core and initiation of infection. Influenza virus mutants which are resistant to high concentrations of amantadine, have hemagglutinin (HA) proteins which fuse with cellular membranes at a higher pH than those of the wild type virus (Daniels *et al.*, 1985). The replication of a number of enveloped RNA viruses, dependent upon receptor mediated endocytosis, are inhibited by amantadine at high concentrations (Wallbank *et al.*, 1966; Helinus *et al.*, 1982).

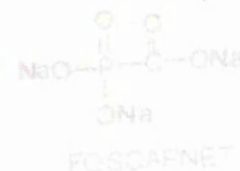
At low amantadine concentrations ($< 5\mu\text{M}$), there is a specific antiviral activity against influenza A virus which operates during both uncoating and particle assembly (Hay *et al.*, (1985). The target of amantadine (at low concentrations) is the M2 protein, a component of the virus envelope (Zabedde and Lamb, 1988). The influenza M₂ protein functions as an ion channel allowing penetration of H⁺ into the virus particle. The decreased pH inside the particle results in the dissociation of the virus matrix protein and the ribonucleoprotein complex which is needed to allow the latter to enter the nucleus and initiate infection (Martin and Helinus, 1991). Amantadine and rimantadine block the M2 ion channel and so inhibit virus particle dissociation (Burkinskaya *et al.*, 1982 and 1982a; Martin and Helinus 1991a).

The M2 protein ion channel also has a role late in the infection cycle of influenza virus. The lumen of cisternae and vesicles of the trans-Golgi network is maintained at an acidic pH by the action of the proton pump. Following influenza virus infection, the M2 ion channel counteracts the low pH by either permitting the outflow of the H⁺ ions or the inflow of counterions. Amantadine induced blocking of the channel results in lowering of pH below that required to trigger the conversion of the native viral protein HA to the low pH form leading to conformational changes in the HA molecule and impairing release of the virus (Sugrue *et al.*, 1990).

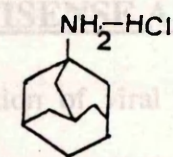
Amantadine-resistant mutants have been easily isolated both *in vitro* (Appleyard, 1977) and *in vivo* (Oxford *et al.*, 1970). Most amantadine or rimantadine resistant isolates of influenza virus have mutations in the M gene that result in a single amino acid substitution in the transmembrane domain of the M2 protein. Mutations in the HA gene have also been reported (Hay *et al.*, 1985).

Amantadine and rimantadine do not have any antiviral activity against influenza B virus (which constitutes 20% of the common influenza infections) and both drugs have been associated with some mild neurological disorders (Dolin *et al.*, 1982). However the drugs have proved efficient in blocking the spread of influenza within isolated small communities (Douglas *et al.*, 1990).

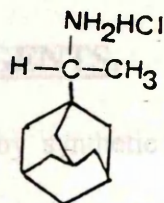
Rimantadine is a licensed therapy for Parkinson disease due to its similarity to L-dopamine.



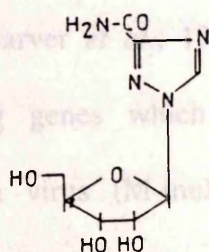
1.1.3. ANTISENSE ANTIVIRAL AGENTS



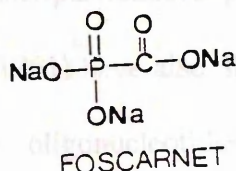
AMANTADINE



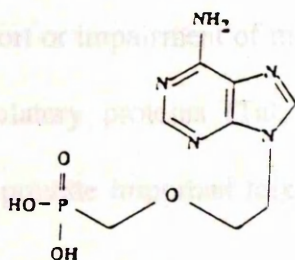
RIMANTADINE



RIBAVIRIN



FOSCARNET



PMEA

FIGURE 2 . Structure of amantadine, rimantadine, ribavirin, foscarnet, and PMEA.

1.1.3. ANTISENSE ANTIVIRAL AGENTS.

Inhibition of viral gene expression by synthetic oligonucleotide analogues with sequences to bind to viral DNA or RNA is a highly specific way to inhibit viral replication.

Antisense oligonucleotide/RNA duplexes can impair viral protein synthesis by the enzymatic hydrolysis of the RNA part of oligonucleotide/RNA duplex by RNase H. An example of the latter group are compounds called ribozymes (Pieken *et al.*, 1991). Ribozymes targeted against HIV-1 gag transcripts reduced p24 protein level in tissue culture (Sarver *et al.*, 1989). Han *et al.*, (1991) have also shown that transgenic mice containing genes which express antisense oligonucleotides against Molony murine leukaemia virus (M-muLV) were resistant to development of leukaemia symptoms following viral infection.

Synthetic oligonucleotides can also specifically interact with mRNA, resulting in inhibition of mRNA transport or impairment of mRNA splicing process (Bischofberger and Wagner, 1992). HIV regulatory proteins (Tat, Rev, and Nef) are essential for virus infectivity, therefore, they provide important targets for antisense oligonucleotides against their mRNA's.

A problem arising from targeting viral mRNA's is the overabundance of the targets. The intracellular concentration of the antisense oligonucleotides required to hybridise with the target RNA's has to be more than the intracellular concentration of the target RNA's. Furthermore, the concentration of the antisense compounds should be kept at steady levels during the therapy as target viral RNA's are continuously being transcribed.

Another mechanism for targeting viral genes is by synthesising oligonucleotides targeted against viral dsDNA (genomic). The advantage of these compounds over the

antisense oligonucleotides against mRNA's, is that there are fewer targets for them to bind. Furthermore, these oligonucleotides can target latent and episomal viral DNA. Binding of these compounds to viral dsDNA forms a triple helix molecule, leading to inhibition of viral replication (Maher *et al.*, 1989). These compound directly target dsDNA viruses.

Eventhough, antisense oligonucleotides offer a very selective range of antiviral compounds with a high therapeutic ratio *in vitro*, several problems *in vivo* have limited their use in therapeutic treatments. These molecules have a very low cellular permeability and are susceptible to degradation by cellular enzymes, furthermore it has been shown that sequences of oligonucleotides which are not expected to bind to any targets have biological activity. This non-specific binding can be against viral or cellular targets.

More work is needed in the area of improving permeability and stability of these compounds *in vivo*, before they can efficiently be used as antiviral agents in therapeutics.

1.1.4. PROTEIN-PROTEIN INTERACTION AS ANTIVIRAL

MECHANISM.

Interaction of viral protein subunits is important for virus replication, therefore any agent which can selectively disrupt this interaction is a potential antiviral agent.

Short synthetic peptides have been shown to selectively disrupt viral protein-protein interactions. Peptides can theoretically inhibit viral protein-protein interaction by competing for the binding site on one of the subunits, bind^{ing} to the interface between the two subunits, or bind^{ing} to a site which will induce conformational changes in the peptides.

1.1.5 Short peptides have been synthesized to identify antiviral targets in different classes of viruses [eg. HSV (Digard et al., 1995; Marsden et al., 1994)]. Short peptides of 15-19 amino acids corresponding to the connection domain of HIV-1 reverse transcriptase have been employed successfully to inhibit dimerization of HIV-1 RT, thus inhibiting reverse transcriptase activity (Divita et al., 1994). However, further modifications are needed to improve delivery and stability of these peptides *in vivo*, before they can be successfully employed in therapeutics.

1.1.6 Aspartic protease inhibitors (1989). Pepstatin A, a specific inhibitor of aspartic proteases has been shown to inhibit retrovirus protease activity at high concentration (Katch et al., 1987).

Many novel inhibitors of HIV protease have been synthesized and several are now in clinical trials [L-735,524 (Merck) (Wei et al., 1995) and AHT 538 (Abbott) (Ho et al., 1995)]. These compounds bind specifically to the active site of the protease resulting in specific conformational changes in structure, and blocking proteolytic activity (Miller et al., 1994).

1.1.7 Zidovudine (AZT) (1987). AZT (3'-azido-2,3'-dideoxythymine) was among the first compounds designed to inhibit HIV-1 protease (Roberts et al., 1990). In cell culture, zidovudine inhibited HIV-1 RT. AZT inhibited the cleavage of the p55 precursor protein into the p24 and p12 products. Antiviral activity was observed at very low concentrations (10⁻⁶ M) of the compound. Antiviral effects occurred only at much higher concentrations (10⁻⁴ M) of the compound.

1.1.8 Zalcitabine (ddC) (1988). ddC (2',3'-dideoxycytidine) was reported in HIV-infected peripheral blood mononuclear cells (PBMCs) to be active in combination with AZT, to 3.0 (Chen et al., 1992).

1.1.9 Zalcitabine (ddC) (1988). ddC (2',3'-dideoxycytidine) was well tolerated by AIDS patients and was reported to be active in combination with AZT, to 3.0 (Chen et al., 1992).

1.1.10 Zalcitabine (ddC) (1988). ddC (2',3'-dideoxycytidine) was well tolerated by AIDS patients and was reported to be active in combination with AZT, to 3.0 (Chen et al., 1992).

1.1.5. ANTI-HIV COMPOUNDS.

PROTEASE INHIBITORS.

The HIV protease is responsible for cleaving the HIV gag and gag-POL polyproteins into their smaller component proteins. Thus, protease activity is essential for the assembly and maturation of the infectious virus. The HIV protease is a member of the aspartic proteases. The protease functions as a dimer with two aspartate amino acids in its active centre (Miller *et al.*, 1989). Pepstatin A, a specific inhibitor of aspartic proteases has been shown to inhibit retrovirus protease activity at high concentration (Katoh *et al.*, 1987).

Many novel inhibitors of HIV protease have been synthesised and several are now in clinical trials [L-735,524 (Merck) (Wei *et al.*, 1995), and ABT-538 (Abbott) (Ho *et al.*, 1995)]. These compounds bind specifically to the active site of the protease resulting in drastic conformational changes in structure, and blocking proteolytic activity (Miller *et al.*, 1989).

RO 31-8959 (saquinavir, Roche) was among the first compounds designed to inhibit the HIV-1 protease (Roberts *et al.*, 1990). In cell cultures chronically infected with HIV-1, Ro 31-8959 inhibited the cleavage of the p55 precursor protein into the p24 and p17 products. Antiviral activity was observed at very low concentrations (1nM), while cytotoxic effects occurred only at much higher concentrations > 5µM. Synergistic effects have been reported in HIV-infected peripheral blood mononuclear cells, when Ro 31-8959 was used in combination with AZT, or ddC (Johnson *et al.*, 1992).

Ro 31-8959 was well tolerated by AIDS patients and was responsible for raising CD4 cell numbers (Vella, 1994). The compound has been licensed as a treatment for AIDS in USA. Rapid degradation of protease inhibitors in human sera, low solubility, and poor

oral bioavailability *in vivo*, have hindered the efficient use of this class of compounds in the treatment of AIDS (Crumpacker, 1992).

HIV-1 mutants resistant to protease inhibitors have been isolated from both *in vitro* and *in vivo* sources. HIV-1 mutants resistant to Ro 31-8959 exhibited amino acid substitutions at positions; 48 (glycine to valine), 90 (Leucine to methionine), and/or 54 (isoleucine to valine) in the protease protein. The most common substitution was at amino acid 48. Virus mutants having all three RO 31-8959 resistance mutations in the protease gene were 50 fold less sensitive than the wild type virus (Eberle *et al.*, 1995).

Viruses resistant to a range of different protease inhibitors have been reported (Condra *et al.*, 1995), and these exhibit a high degree of cross-resistance between the different inhibitors (Tisdal *et al.*, 1995).

AZIDOTHYIMIDINE(AZT),3'-AZIDO-3'-DEOXYTHYIMIDINE)/

ZIDOVUDINE/RETROVIR.

Azidothymidine was first synthesised, as an anti-cancer drug in 1964 by Horwitz *et al.*, and was the first drug to be licensed for use in the treatment of AIDS patients (Figure 3). AZT is converted to its active form (AZT-triphosphate) by cellular kinases (Furman *et al.*, 1986). Thymidine kinase converts AZT to its monophosphate and diphosphate forms, while thymidylate kinase converts it to AZT-triphosphate (Furman *et al.*, 1986). AZT-triphosphate is a very potent inhibitor of the HIV-1 reverse transcriptase enzyme, with an affinity 100-fold greater for HIV-RT than cellular polymerases (Furman *et al.*, 1986). AZT-TP competes with dTTP as substrate for the HIV-RT. When AZT is incorporated into the viral growing DNA chain, it causes DNA chain termination. AZT-TP cannot form a

normal 3'- to 5'- phosphodiester bond with the hydroxyl group (5'-OH) of the next nucleotide, since it lacks the 3'-OH group on the ribose sugar (Furman *et al.*, 1986; Mitsuya and Border, 1986 and 1987)

In the first clinical trials conducted by (Fischl *et al.*, 1987), it was revealed that treatment with AZT resulted in a reduction in HIV p24 antigen, a moderate increase in the CD4 counts, and a significant reduction in the incidence of death among patients in the test group. This study also revealed some of the toxic-side effects of AZT treatment, including; headaches, nausea, insomnia, myalgia, and most importantly haematological disorders (anaemia, neutropenia). Following confirmation by other clinical trials, AZT became the drug of choice in combating AIDS. Recent results from the Concord studies have revealed that the administration of AZT early in HIV infection has no significant effect on the rate of survival of the patients or the progression of HIV disease. Although a transient clinical benefit was observed in the test group.

Prolonged treatment of AIDS patients with AZT is frequently associated with the emergence of , AZT-resistant mutants (Larder *et al.*, 1989). A further study by Larder *et al.*, (1989a), indicated that amino acid substitution in the HIV reverse transcriptase protein at positions; 67 (Asp to Asn), 70 (Lys to Arg), 215 (Thr to Phe or Tyr), and 219 (Lys to Gln) were responsible for the resistance to AZT. More recent study has identified an additional substitution at position 41 (Meth to Leu) which also confers resistance to AZT (Kellam *et al.*, 1992).

DIDEOXYCYTIDINE (ddC) AND DIDEOXYINOSINE (ddI).

2', 3'-dideoxycytidine (Zalcitabine) and 2', 3'- dideoxyinosine (Didanosine) (Figure 3) are nucleoside analogues with a mode of action similar to that of AZT (Cooney *et al.*, 1986; Mitsuya and Broder, 1986). HIV isolates resistant to ddI and ddC have been isolated from patients after prolonged drug treatment (St.Clair *et al.*, 1991). DdI resistance was generated by a point mutation in the HIV RT gene resulting in a Leu to Val change at amino acid 74. Curiously, this same mutation resulted in increased sensitivity to AZT (St. Clair *et al.*, 1991). Combinations of ddI or ddC with AZT have been administered for the treatment of AIDS patients (particularly those suffering from AZT-induced suppression of bone marrow activity). *In vitro* experiments using AZT and ddI in combination, resulted in synergistic antiviral activity but only additive cytotoxicity (Dornsife *et al.*, 1991). The exact mechanism by which the synergistic effect is achieved is not known. However, when AZT and ddI are combined at the ratio of 1:1 the phosphorylation of ddI to the active triphosphate form was increased (Palmer and Cox, 1994). More recently, however, it was demonstrated that AZT and ddI could be mixed at ratios other than 1:1, and still exhibit synergistic antiviral activity (Palmer and Cox, 1995).

NEVIRAPINE.

5,11-dihydro-11-cyclopropyl-4-methyl-6H-dipyrido-[3,2-b2',3'e][1,4]diazepin-6-one (Nevirapine) (BI-RG-587) (Figure 3) is a dipyridodiazepinone and a non-nucleoside inhibitor of HIV-RT (Merluzzi *et al.*, 1990). Based on studies with HIV infected and uninfected human T-cells the K_i of nevirapine for HIV-RT was reported to be 200nM, with a therapeutic index of > 8000 (Merluzzi *et al.*, 1990).

The drug is a specific inhibitor of HIV-RT and operates by binding non-competitively (with respect to the primer, template, nucleotide, and RNA) to the tyrosine residues at amino acid position 181 and 188 of the p66 subunit of HIV-1 RT enzyme (Kohstaedt *et al.*, 1992 and Shih *et al.*, 1991). Nevirapine also partially inhibits the RNase activity of the RT (Merluzzi *et al.*, 1990). Binding of nevirapine to RT is thought to result in conformational changes, which affect the polymerase activity more than the RNase activity. The activity of nevirapine is highly specific for HIV-1 RT and has no effect on SIV RT, or FeLV-A RT, nor does it affect the activities of human DNA polymerases α , β , γ , or δ (Merluzzi *et al.*, 1990). The IC₅₀ concentrations of nevirapine against different laboratory strains of HIV-1 ranged from 32 to 42 nM. However the compound has no activity against HIV-2 strains (Merluzzi *et al.*, 1990).

At concentrations 8000-fold lower than the cytotoxicity dose, nevirapine inhibited HIV-1 induced syncytium formation and p24 antigen production by wild type virus in tissue culture experiments (Richman *et al.*, 1991). Nevirapine is synergistic in combination with AZT, and also inhibits AZT-resistant HIV-1 mutants (Richman *et al.*, 1991). A clinical trial involving 21 HIV-infected patients, showed that, treatment with nevirapine at concentrations up to 400mg/day was well tolerated (Cheeseman *et al.*, 1992). Results from phase I and II clinical trials of nevirapine monotherapy showed a reduction in serum p24 levels and an increase in the CD4 cell counts, after only 7 days treatment (Cheeseman *et al.*, 1992). However, the p24 and CD4 values returned to their initial values shortly after discontinuation of the therapy. Nevirapine-resistant viruses can be isolated after a very short time (as early as a week after drug treatment) (Richman *et al.*, 1994). The nevirapine resistant mutant most commonly isolated after nevirapine monotherapy has a

tyrosine to cysteine substitution at residue 181 in the RT protein (Richman *et al.*, 1991a and 1994).

TIBO.

Tetrahydroimidazol[4,5,1-jk][1,4]-benzo-diazopin-2(1H)thione (TIBO) (Figure3), is another non-nucleoside inhibitor of HIV-1 reverse transcriptase activity (Pauwels *et al.*, 1990). The mechanism of the action of TIBO is very much the same as nevirapine, indeed nevirapine is inactive against TIBO-resistant mutants (Richman *et al.*, 1991a).

TIBO completely inhibited p24 production from HIV-1 infected peripheral blood lymphocytes at 0.7 μ M, whereas a cytotoxic effect was observed at 800 μ M (Pauwels *et al.*, 1990). TIBO selectively inhibits the HIV-1 RT polymerase activity and has no effect on the RNase activity of the enzyme, and no activity against HIV-2 RT activity. Neither do the drugs inhibit human DNA polymerases α , β , or γ activities (Debyser *et al.*, 1991; Frank *et al.*, 1991). Treatment of HIV-infected cell cultures with TIBO resulted in rapid emergence of TIBO-resistant mutants [with a single base mutation at codon 181 (Tyr to Cys)] (Larder, 1992). This mutant was cross-resistant to nevirapine. Furthermore, acquisition by AZT-resistant isolates, of TIBO resistance mutation at position 181, resulted in a suppression of AZT resistance (Larder *et al.*, 1992).

TAT INHIBITORS.

[7-chloro-5-(2-pyrryl)-3H-1,4-benzodiazepine-2(H)-one] (Ro 5-3335) and its derivative [7-chloro-N-methyl-5 (1H-pyrrol-2-yl)3H-1,4-benzodiazepin-2-amine] (Ro 24-7429) are both potent inhibitors of TAT-mediated transcriptional activation in HIV-1 (Hsu

et al., 1991 and 1993). Ro 24-7429 toxicity in laboratory animals is less than that of Ro 5-3335.

While the mechanism of action of these compounds against HIV-TAT is not fully understood, it is possible that they impair TAT binding to TAR (the cis acting binding site for TAT). Alternatively they might inhibit interaction of TAT with cellular factors (Hsu *et al.*, 1993).

Ro 24-7429 is active against a broad range of HIV-1 laboratory strains, and is also active against HIV-2 Tat activity. The drug inhibited viral RNA and antigen production in both acutely and chronically infected cell cultures at noncytotoxic concentrations (IC₅₀ at 0.2-1 μ M). Ro 24-7429 was also effective against clinical isolates resistant to AZT and nevirapine (Hsu *et al.*, 1993). Following two years of repeated weekly passages of the virus in the cell culture in the presence of the RO 24-7429, no resistant virus mutants have been isolated. Despite of these encouraging results, Ro 24-7429 proved to have low solubility *in vivo*, and the results from phase I and II clinical trials have indicated that RO 24-7429 is inefficient *in vivo* (Bowen *et al.*, 1995)

1.1.5.1. ANTI-HUMAN IMMUNODEFICIENCY VIRUS COMPOUNDS DERIVED FROM NATURAL SOURCES.

Despite the immense effort expended in pursuing rational drug design programmes, there remains a need to investigate the vast reservoir of natural compounds for those that display antiviral activity. Hundreds of thousands of terrestrial and marine plant extracts have been tested for activity against HIV by both the US National Institute of Health (NIH), and pharmaceutical companies. Because plants or plant extracts are used in traditional

medicine throughout the world to treat a variety of diseases, including viral infections, the World Health Organization (WHO) has encouraged scientific investigation of plant extracts for possible anti-HIV activity.

Although many plant derived compounds have been administered to AIDS patients in under-developed countries, there is either no scientific basis for their use, or if there is a

genuine anti-HIV activity, the antiviral mechanism remains obscure. Some of the naturally occurring compounds which have shown anti-HIV activity are described below.

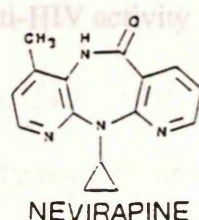
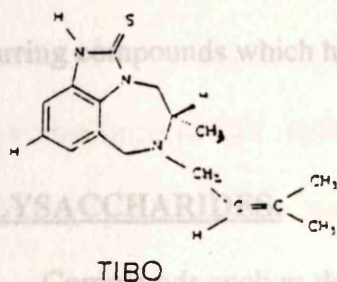
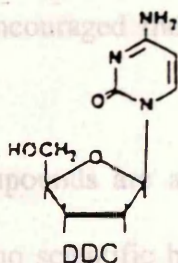
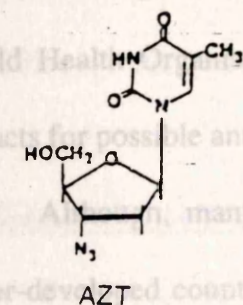


FIGURE 3 . Stucture of anti-HIV compounds.

Compounds such as dextran sulphate and heparin (Figure 4) block the adsorption of HIV to the cell membrane (reviewed by Wright, 1995). Both of these compounds completely protected MT-4 cells in culture against HIV-1 induced cytopathogenicity at 25µg/ml, while no cytotoxicity was observed at 625µg/ml (Baba *et al.*, 1988). The compounds are stable at concentrations that are thought to be safe in humans (eg. heparin and dextran sulphate inhibit HIV replication at 100 and 1700 of concentration required for their anti-coagulant effect respectively). However, poor oral absorbance and low stability have limited their use as an effective anti-HIV treatment (reviewed by Wright, 1995).

PSEUDOMONAS EXOTOXIN.

A novel approach has been taken in delivering toxic compounds to HIV infected cells. A recombinant protein consisting of the CD4 molecule and the pseudomonas exotoxin (PE) has been developed. This protein is able to bind to the HIV receptor and deliver the toxic PE into the cell.

medicine throughout the world to treat a variety of diseases, including viral infections, the World Health Organisation (WHO) has encouraged the scientific investigation of plant extracts for possible anti-HIV activity.

Although, many plant derived compounds are administered to AIDS patients in under-developed countries, there is either no scientific basis for their use, or if there is a genuine anti-HIV activity, the antiviral mechanism remains obscure. Some of the naturally occurring compounds which have shown anti-HIV activity are described below.

POLYSACCHARIDES.

Compounds such as dextran sulphate and heparin (Figure 4) block the adsorption of HIV to the cell membrane (reviewed by Wright, 1995). Both of these compounds completely protected MT-4 cells in culture against HIV-1 induced cytopathogenicity at 25µg/ml, while no cytotoxicity was observed at 625µg/ml (Baba *et al.*, 1988). The compounds exhibited anti-HIV activity at concentrations that are thought to be safe in humans (eg. heparin and dextran sulphate inhibit HIV replication at 1/10 and 1/100 of concentration required for their anti-coagulant effect respectively). However, poor oral absorbance and low stability have limited their use as an effective anti-HIV treatments (reviewed by Wright, 1995).

PSUEDOMONAS EXOTOXIN.

A novel approach has been taken in delivering toxic compounds to HIV-infected cells. A recombinant protein consisting of the CD4 molecule and the pseudomonas

exotoxin-A, selectively destroys HIV infected cells by binding to the, HIV surface protein (gp120), expressed on their plasma membrane (Chaudhury *et al.*, 1988).

ALKALOIDS AND SUGAR ALKALOIDS.

Papaverine is an alkaloid present in the seeds of the opium poppy *Papaver somniferum* (Figure 4). This compound is already used clinically as a smooth muscle relaxant for the treatment of gastrointestinal colic. Turano *et al.*, (1989) have shown that Papaverine impairs HIV replication *in vitro*; viral protein synthesis was markedly reduced although, not all proteins were equally affected (the synthesis of envelope proteins was most markedly inhibited). However, in clinical trials the serum level of HIV-core protein (p24) was not affected by papaverine treatment, although opportunistic infections were decreased and lymphocyte counts improved (Reviewed by Wright *et al.*, 1995).

Sugar alkaloids, in which a nitrogen has replaced the oxygen atom on the sugar molecule generally exert an antiviral effect by inhibiting protein glycosylation. Castanospermine [from the seeds of the Australian Moreton Bay chestnut tree (*castanospermum australe*)] (Figure 4), inhibits α -glucosidase I, thereby preventing the trimming of glucose residues; an early event in glycoprotein processing (Elbein *et al.*, 1984; Saul *et al.*, 1985).

Deoxyojirimycin, a sugar alkaloid present in *Bacillus* and *Streptomyces* bacteria, and also in the berry of *Morus* (mulberry) (Reviewed by Wright *et al.*, 1995) inhibits both glucosidase I and II activities (Furmann *et al.*, 1985). Both castanospermine and deoxyjirimycin have been shown in *in vitro* experiments to impair syncytium formation and virus replication in HIV-infected cells at concentrations of $\geq 1\mu\text{M}$ (Gruters, *et al.*, 1987;

Tyms *et al.*, 1987; Walker *et al.*, 1987). The effect of castanospermin on HIV induced syncytium formation is probably due to the inhibition of processing of the HIV glycoprotein precursor gp160. HIV-infected T-cells treated with castanospermine exhibited reverse transcriptase activity and produced capsid protein (p24) at the same levels as control cells, although, infectious virus yields were reduced (Walker *et al.*, 1987). *In vitro* experiments have shown that, while the anti- HIV-1 and HIV-2 activities of castanospermine and AZT was synergistic, cytotoxicity was unaffected (Johnson *et al.*, 1989). Although, both castanospermine and deoxyjirimycin are well tolerated in tissue culture, little, or no information regarding the toxicity *in vivo* has been available.

FLAVONOIDS AND TANNINS.

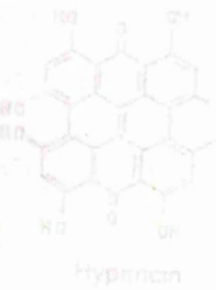
While, flavanoids such as quercetin, quercetagenin, baicalein (Figure 4) and myricetin all have anti-RT activity, the use of these compounds as anti-HIV drugs is excluded by the finding that they are also potent inhibitors of mammalian DNA polymerases (reviewed by Wright, 1995)

AROMATIC POLYCYCLIC DIONS.

Hypericin and psuedohypericin are aromatic polycyclic dions (Figure 4), isolated from plants of the *Hypericum* family. Both compounds have anti-retrovirus activity; inhibiting the replication of Friend leukaemia virus (FV), and radiation leukaemia virus (RadLV), both *in vitro* and *in vivo* (Meruelo *et al.*, 1988). Treatment of FV-infected BALB/c strain mice with a single dose of either ^{of} the compounds, prevented virus-induced

death. The *in vitro* studies suggest that the mode of antiviral action is likely to be through interference with the assembly, maturation or release of the virus (Meruelo *Et al.*, 1988).

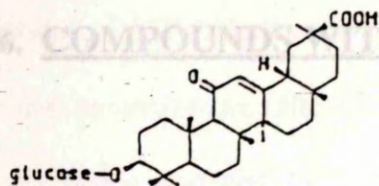
Glycyrrhizic acid



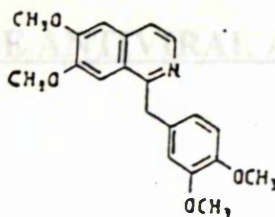
Quercetin

FIGURE 4. Structure of anti-HIV compounds derived from natural products.

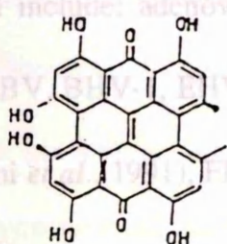
1.1.6. COMPOUNDS WITH BROAD-RANGE ANTIVIRAL ACTIVITY



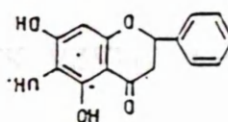
Glycyrrhizic acid



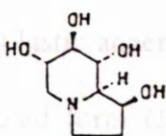
Papaverine



Hypericin



Baicalein



Castanospermine

FIGURE 4 . Stucture of anti-HIV compounds derived from natural sources.

1.1.6. COMPOUNDS WITH BROAD-RANGE ANTIVIRAL ACTIVITY.

PMEA (ADEFOVIR).

9-(2-phosphonylmethoxyethyl) adenine (PMEA) (Figure 2) and its derivative, 9-(2-phosphonylmethoxyethyl)-2,6-diaaminopurine (PMEDAP) are potent and selective antiviral agents having broad-range antiviral activity (De Clercq, 1991). The viruses inhibited include; adenovirus (Baba *et al.*, 1987), herpesviruses [HSV-1, HSV-2, VZV, CMV, EBV, BHV-1, EHV-1, TK⁻ HSV-1, and TK⁻ VZV] (De Clercq *et al.*, 1986), HIV-1 (Balzarini *et al.*, 1991), FIV (Egbernic *et al.*, 1990), and Friend leukaemia virus (Naesens *et al.*, 1993).

PMEA blocked FIV replication (ED₅₀ 0.6μM) in infected feline thymocytes, and suppressed virus replication in FIV-infected cats. However, at very high doses of the drug, toxic side effects, such as megaloblastic anaemia occurred (Egbernic *et al.*, 1990). PMEA is converted to its diphosphorylated form (PMEA-DP), by cellular 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase (Balzarini *et al.*, (1991). The affinity of PMEA-DP for HIV-1-RT is higher than that for cellular DNA polymerase (K_i/K_m values of 0.01 and 0.6 respectively). PMEA-DP competes with dATP for HIV RT. PMEA-DP is an inhibitor of RT and, after incorporation into viral DNA, results in DNA chain termination (Balzarini *et al.*, 1991). The oral bioavailability of PMEA is low (12%) (Cundy *et al.*, 1995). However, *in vivo*, PMEA-DP has an intracellular half life of 16-18h (Balazarini *et al.*, 1991). Thus, in contrast to AZT, PMEA required less frequent administration to be effective against HIV. PMEDAP, however, proved to have good oral bioavailability in mice (Naesens *et al.*, 1993). A study by Valenkamp *et al.*, (1995) comparing PMEA and PMEDAP in the

treatment of FIV infected cats, revealed that the latter inhibited FIV replication (both *in vivo* and *in vitro*) more efficiently and was less toxic.

PMEA and PMEDAP have both anti-herpes and anti-retrovirus activity, and this could be beneficial in the treatment of AIDS patients in who, herpesviruses virus infections are very common.

RIBAVIRIN (VIRAZOLE).

Ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (Figure 2) is a synthetic nucleoside analogue, first synthesised in 1972, and shown to have antiviral activity against a wide range of DNA and RNA viruses *in vitro* (Sidwell *et al.*, 1972).

The precise antiviral mechanism of the drug is still not clear. Ribavirin is phosphorylated by cellular enzymes to ribavirin-TP. Ribavirin-MP inhibits inosine monophosphate dehydrogenase (IMD), which is responsible for the synthesis of GMP from IMP, resulting in a decreased pool of GTP, inhibiting both DNA and RNA synthesis (Streeter *et al.*, 1973). Ribavirin-TP, however, interferes with the capping and elongation of m-RNA's. The antiviral activity of ribavirin-TP against influenza virus is thought to be due to inhibition of the mRNA capping enzyme, guanyltransferase (Wray *et al.*, 1985). The multi-site antiviral action of ribavirin might explain, the failure to isolate ribavirin-resistant mutants (Gilbert *et al.*, 1986).

In 1986, the US Food and Drug Administration (FDA) approved the use of ribavirin aerosol for the treatment of respiratory syncytial virus (RSV) infection in infants.

FOSCARNET.

Trisodium phosphonoformic acid (Foscarnet) (Figure2) is a pyrophosphate analogue. Foscarnet inhibits the replication of a diverse range of DNA and RNA viruses by directly blocking the pyrophosphate binding site on the POL protein leading to inhibition of polymerase activity (Chrisp and Clissold, 1991). Since the mechanism of antiviral activity of foscarnet is different than that of ACV or GCV, it is currently used for treatment of ACV-resistant and GCV-resistant herpesvirus infections (Field *et al.*, 1994). Foscarnet has also been licensed for the treatment of retinitis caused by HCMV in AIDS patients (Field *et al.*, 1994). HCMV isolates resistant to foscarnet and having point mutations in the DNA polymerase gene have been reported (Sullivan and Coen, 1991).

In a recent study, in which AIDS patients were treated with foscarnet for one week, a reduction in serum p24 level was found in all of the patients (Fletcher *et al.*, 1994). Inhibition of HIV reverse transcriptase by foscarnet was probably responsible for the finding. After long-term treatment of AIDS patients with foscarnet, the emergence of HIV foscarnet-resistant isolates was rare (Tachedjian *et al.*, 1994), but was associated with point mutations in the RT gene. (Mellors *et al.*, 1995).

Unfortunately, foscarnet is accumulated in bone tissue. Although it does not suppress bone marrow activity, it has been associated with renal failure (Chrisp and Clissold, 1991).

1.1.7. TRITERPENOIDS.

The aqueous extract of licorice root (*Glycyrrhizia Radix*), has been widely used in traditional Chinese medicine for its anti-inflammatory properties. Knowledge of the medicinal properties of licorice root has a long history, and the work of Hippocrates published in the 5th century BC recorded its use as a therapeutic agent (Fujisawa, 1993). Glycyrrhizin (GL), a saponin present in the aqueous extract of licorice root, was first isolated in 1909 and a derivative, carbenoxolone sodium (CBX), became the first compound used for the treatment of gastric ulcer in the 1960 s. GL consists of one molecule of the triterpene, glycyrrhetic acid (also known as enoxolone) and two molecules of glucuronic acid (Arai, 1981). The enoxolone portion of the compound is thought to constitute the active part of the molecule. A family of related compounds based on enoxolone have been produced. The structural formulae of glycyrrhizin (GL), enoxolone (EX), and the two synthetic derivative investigated in this thesis; carbenoxolone sodium (CBX) and cicloxolone sodium (CCX), are shown in Figure 5. Triterpenoid compounds are highly lipophilic, bind strongly to cellular membranes (Johnston *et al.*, 1974), and at high drug concentrations ($>50\mu\text{M}$ CCX) can cause the plasma membrane⁵ to become leaky (Dargan and Subak-Sharpe, 1986; Reardon and Audus, 1993). Triterpenoid compounds are also known to disturb the normal flow of monovalent cations across plasma membrane (Baron and Green, 1986).

Pompei *et al.*, (1979, 1980 and 1981) have investigated the antiviral activity of GL against a range of DNA and RNA viruses, and have found that at GL concentrations well tolerated by cell cultures (2mM and 4mM) the growth of vaccinia, HSV, Newcastle disease virus (NDV), and vesicular stomatitis virus (VSV) was inhibited. In addition, GL inhibited

the replication of influenza (A and B) and NDV in embryonated eggs (Pompei *et al.*, 1983). Baba and Shigeta (1987) have reported that GL has anti-VZV activity. GL induced inhibition of VZV replication in HEF cells operated at an early stage in the virus replication cycle (penetration or uncoating). These authors also reported that GL in combination with other compounds (acyclovir, adenine arabinoside, bromovinyldeoxyuridine or phosphonoformate) resulted in additive or slightly synergistic antiviral activity in *in vitro* experiments.

The US food and drug administration (FDA) has approved GL for human use. Taken in sufficient quantity, triterpenoid compounds, can induce a mineral-corticoid effect (increased serum sodium level and decreased serum potassium level) (Tomkins and Edmond, 1975; Epsteine *et al.*, 1977) (this may also be induced by the consumption of confectionery licorice). The basis for the mineral corticoid activity is thought to result from the effect of triterpenoid compounds on the cellular $\text{Na}^+/\text{K}^+\text{ATPase}$ (the sodium pump). Baron and Green (1986) have shown that CBX stimulates $\text{Na}^+/\text{K}^+\text{ATPase}$ activity in intact human leukocyte.⁵ Using ouabain, a specific inhibitor of $\text{Na}^+/\text{K}^+\text{ATPase}$, Gary *et al.*, (1979) have shown that the increase in enzyme activity was not due to the synthesis of new enzyme molecules or unmasking of presynthesized molecules, but to a direct stimulation of the operation of pre-existing sodium pumps. The sodium pump hydrolyses ATP to exchange 2 intracellular Na^+ ions for 3 extracellular K^+ ions. The $\text{Na}^+/\text{K}^+\text{ATPase}$ enzyme consists of two separate proteins which function as α and β heterodimers. The catalytic activity of the sodium pump is associated with the α subunit. The α subunit of the sodium pump is regulated through the action of protein kinase C (PKC) (Ling *et al.*, 1984; Bertorrelo *et al.*, 1991; Middleton *et al.*, 1993). $\text{Na}^+/\text{K}^+\text{ATPase}$

activity is inhibited by PKC-mediated phosphorylation of the α subunit of the pump. Other cell-response factors, such as neurotransmitters and hormones, which trigger PKC activity also inhibit Na^+/K^+ ATPase activity (Bertorello *et al.*, 1991).

Ito *et al* (1987) have demonstrated that GL inhibits the activity of the PKC. Consequently, it seems probable that the observed triterpenoid-induced increase in Na^+/K^+ ATPase activity is due to impaired regulation of the sodium pump via decreased phosphorylation of the α -subunit of the enzyme by PKC. Although, this seems a likely explanation, it should be noted that, the Na^+/K^+ ATPase present in some cell types does not respond to PKC (Middelton *et al.*, 1993) (this may explain the differential tolerance of various cell lines for triterpenoid drugs). Furthermore, it may also be relevant to note that PKC is a ubiquitous enzyme that exists in several different soluble and membrane-bound isoforms, some of which are involved in secretion, exocytosis, modulation of ion conductance, regulation of receptor interaction with components of the signal transduction apparatus, gene expression, and cell proliferation (Nishizuka, 1986 and 1988). Thus it is possible that the triterpenoid compounds interfere with several different cellular biochemical systems.

Prolonged triterpenoid treatment, especially in patients of advanced years, may induce psuedoaldosteronism (increased serum Na^+ level) and hypokalaemia (decreased serum K^+ level) (Stewart *et al.*, 1987). These unwanted side-effects can usually be controlled by treatment with diuretics and are reversible upon removal of the drug (Molhuysen *et al.*, 1950; Conn *et al.*, 1968). In order to alleviate the *in vivo* mineral-corticoid effect of GL, an intravenous preparation of GL, consisting of GL (0.2%), Cysteine (0.1%), and glycine (2 %) in physiological saline, sold under the name of Stronger Neo-

Minophagen C (SNMC) is routinely used in Japan to treat chronic viral hepatitis and/or allergic skin diseases (Fugisawa *et al.*, 1980; Suzuki *et al.*, 1983). The glycine present in SNMC is said to eliminate the mineral-corticoid effect of GL, while the cysteine is thought to have detoxicative and anti-allergic properties.

The triterpenoid compounds can be cytotoxic in tissue culture, but the magnitude of the cytotoxic effect depends upon the type of the cell line used. Based on their tolerance for CCX, Galt *et al.*, (1990) have classified ~ 20 cell lines, into three different groups; resistant (CCX^r), intermediate in sensitivity (CCX^I), and sensitive (CCX^s). The cell lines classified as CCX-resistant included: bovine MDBK, canine MDCK, rabbit RK, and human; Hela, Hep2, Chang liver, Detroit 532, and Flow 2002 cells. The intermediate class contained; rabbit epithelium, rabbit keratocytes, hamster BHK 21, human MRC-5, and human flow 4004 cells. The CCX-sensitive class included; rat cells (Re 99, Re α , and Hood cells), primary chicken embryo fibroblast, human FG293, and frog XTC-2 cells. The cell type may determine tolerance to CCX; of 10 fibroblastic cell lines two belonged to the resistant class, three were placed in the intermediate group, and the rest belonged to the sensitive class, while 7 of 11 epithelial cell lines examined were classified as resistant (Galt *et al.*, 1990). CCX-resistant cell lines have facilitated the study of the antiviral activity of the drug because the cytotoxic and the antiviral effects can be uncoupled.

A randomised double-blind placebo controlled trial of 133 chronic active hepatitis patients (diagnosed by histological techniques) treated intravenously with 80 mg of GL (SNMC) per person/day for one month, showed that there was a significant improvement in serum transaminase levels in the test group compared to those of the placebo group (Suzuki *et al.*, 1983). Several clinical trials in Germany (between 1988-1991) have shown that

treatment of patients, diagnosed as having a history of chronic active hepatitis B and C with SNMC [usually three 60 ml (80 mg of GL) injections/week] for a period of 6 months to 3 years, resulted in significant improvement in liver transaminase enzymes (AST, and ALT) in more than 80% of the patients. Importantly, no side effects were reported, nor were relapses observed after withdrawal of treatment (Wildhirt, 1993). The mechanism by which GL exerts its anti-hepatitis B activity is not clear. It may be due to the induction of interferon by the drug (Abe *et al.*, 1982) or may be due to a direct effect of the drug on virus replication.

Takahara *et al.*, (1994) have shown that glycyrrhizin suppresses the secretion of the hepatitis B surface antigen (HBsAg) in PLC/PRF/5 cells in culture, resulting in the accumulation of the HBsAg protein in cytoplasmic vacuoles located at, or near the proximity of the Golgi apparatus. GL suppressed the intracellular transport of HBsAg at a stage after O-linked glycosylation but before sialation of the protein; probably in a trans-Golgi compartment.

GL also inhibited replication of hepatitis A virus (HAV) in PLC/PRF/5 cells; this was not due to virucidal activity against HAV, since GL had no effect on the adsorption of (³H) uridine-labelled HAV to the cells (Crance *et al.*, 1995). The same group of workers, also showed that a combination of GL and interferon α resulted in a synergistic antiviral effect against HAV.

Treatment of infants exhibiting abnormal liver function caused by congenital CMV infection, with SNMC (60 mg of GL) (50ml/day for a period of one week), resulted in recovery of normal liver function, indicating possible anti-CMV activity for GL (Numazaki

et al., 1994). Indeed, Galt *et al.*, (1990) have demonstrated *in vitro* anti-HCMV activity for CCX.

The anti-HIV activity of GL (SNMC) has been demonstrated both *in vitro* and *in vivo*. GL completely inhibited the HIV-induced fusion of MT-4 cells and HIV antigen expression, at 0.3 and 0.6mM respectively. The GL ID₅₀ for HIV in MOLT 4 cells was determined to be 0.15mM (Ito *et al.*, 1987). Studies of the mode of anti-HIV activity of GL revealed that, GL did not impair HIV-1 reverse transcriptase activity in *in vitro* assays, even at concentrations up to 12 mM (Ito *et al.*, 1987). The observation that treatment of HIV-1 infected cell cultures with a combination of GL and AZT resulted in additive antiviral activity, implies that the antiviral targets of these two compounds are different, and that the target for GL is not RT (Ito *et al.*, 1987). Further studies by Ito *et al.*, (1988) have shown that GL inhibits protein kinase C (PKC), a ubiquitous enzyme responsible for phosphorylation of the CD4 protein on the cell surface. The CD4 protein is recognised as the specific receptor for HIV on susceptible lymphocyte cells. The authors suggested that at least part of the anti-HIV mechanism of GL operates at the level of virus adsorption (through the inhibition of CD4 phosphorylation). The effect of GL on virus binding can only account, however, for a relatively small contribution to the overall antiviral effect of GL, indicating that other antiviral mechanisms are also in operation. In contrast to the findings of Ito *et al.*, (1988), Ohtsuki *et al.*, (1988) could find no evidence that GL inhibited PKC, or protein kinase A activity but did report that low doses of GL selectively inhibited the activity of protein kinase P in a dose-dependent manner. As PKC exists in up to eleven different isotypes, it is possible that these conflicting reports may be due to the use of different lines by each group of investigators.

Several groups have investigated the treatment of AIDS patients with GL and have reported some clinical benefit. Hattori *et al.*, (1989) reported that AIDS patients (with T4 lymphocyte count of $< 100/\mu\text{l}$ of serum) treated with intravenous SNMC (at 400-1600mg of GL/day, for a month), had a reduced level of serum HIV p24 antigen. However, a sustained increase in the number of T4 cells was not achieved. An increase in T8 cell number was observed and was thought to be due to the immunostimulatory effect of GL (Kimura *et al.*, 1992). GL stimulates interferon γ production in mice (Abe *et al.*, 1982), and enhances both antibody production and cellular immune responses such as; lymphocyte proliferation, interleukin 2 synthesis, and natural killer activity (Kakumu, 1993). Treatment of nine haemophiliac patients diagnosed as HIV-positive (asymptomatic carriers), with intravenous SNMC (200-800mg GL /day for more than 8 weeks), resulted in an increase in T4 cell numbers in all cases, and an increase in the CD4/CD8 cell ratio for 6 out of 9 cases (Mori *et al.*, 1989). As expected, treatment of 3 control patients (HIV-negative haemophiliacs) with GL did not alter the number of T4 lymphocytes (Mori *et al.*, 1989). The authors, therefore suggested that treatment of HIV-positive individuals with large doses of GL early in HIV-1 infection, might delay or prevent the infection from developing into full blown AIDS (Mori *et al.*, 1990).

The two derivatives of GL; CBX and CCX have been shown in clinical trials to be effective against orofacial and genital HSV infection (Poswillo and Roberts, 1981; Csonka and Tyrrell, 1984; Partridge *et al.*, 1984).

Triterpenoid compounds exhibit broad range antiviral activity. It is thought that antiviral activity of the compounds does not stem from impairment of any specific virus gene or virus gene product, but rather that the drugs inhibit virus replication by disturbing

cell membrane functions which are essential for virus replication (Dargan *et al.*, 1992). Based on sensitivity to CCX, DNA and RNA viruses from eight different families were placed into three classes (Galt *et al.*, 1990). The first class, CCX-sensitive 1 (CCX^s-1) (100-100,000 fold loss of infectivity in the virus yield) was composed of; HSV-1, HSV-2, EHV-1, BHV-1, VSV, and FLU A. Based on their ED50 values, VZV and HCMV were also included in this group. The dose-response curves of this class were characterised by a continuous CCX-dose-dependent decrease in virus yield. The second class (CCX^s-2) consisted of; Ad-5, Reo-3, polio-1, and the bunyaviruses (Germiston, and Bunyamwera). The dose-response curves of this class were characterised by bi-phasic kinetics; with increasing concentration of CCX, there was an initial drop in the virus yield (10-fold to 100-fold reduction in infectious virus yield), after which the curve reached a plateau. The third class (CCX^s-3), contained only one virus, the togavirus (SFV). The overall yield of infectious SFV was not affected by treatment with up to 300µM CCX, although the release of the virus from infected cells was impaired. The sensitivity of different viruses to CCX did not correlate with virus genome type (DNA or RNA; single-stranded or double-stranded; segmented or non-segmented RNA genome). Neither was there any indication that enveloped viruses were more CCX sensitive than non-enveloped viruses (Galt *et al.*, 1990).

The plateau reached in the dose-response curves of the CCX^s-2 group viruses was not due to the emergence of drug-resistant viruses, but was thought to be either due to the partial impairment of a process essential for virus infectivity or to inhibition of a cell or virus specific function which modifies, but is not essential for virus infectivity (Dargan *et*

al., 1992). That a virus specific component can contribute to the overall antiviral effect of the drug was shown by the finding that, the difference in the CCX-sensitivity of HSV-1 and HSV-2 mapped to genes encoding glycoproteins; UL22 (gH) and UL44 (gC). Differential sensitivity of both viruses to monensin mapped to the same genome locations and suggest that monensin and CCX share the same antiviral target - the Golgi apparatus. (Dargan and Subak-Sharpe, 1991). Repeated attempts to isolate HSV-1, HSV-2, and VSV variants resistant to CCX were unsuccessful (Dargan and Subak-Sharpe, unpublished results). The proposed mode of antiviral action of the drug, suggests that, drug-resistant mutants would be unlikely to emerge.

The overall antiviral activity of CCX is composed of three effects. The particular combination of these effects and their magnitude was found to vary between the different viruses tested. The first effect arises from poor quality (low infectivity) progeny virus produced in the presence of the drug. Dargan and Subak-Sharpe (1985) showed that the particle/p.f.u. ratio of HSV increased by 100-fold to 1000-fold in CCX-treated infected cell cultures. EM studies revealed that, although DNA-containing HSV-nucleocapsids were produced in the presence of CCX, their maturation into fully infectious virion was inhibited (Dargan *et al.*, 1988). The effect of CCX on progeny particle quality (infectivity) might be explained by the effect of the drug on virus protein synthesis and post-translational processing, and/or by the incorporation of the drug molecule into the virion envelope. CCX treatment resulted in the assembly of HSV particles with a protein composition different from that of control HSV particles. Both the amounts of individual proteins and their apparent molecular weights were altered. CCX and CBX treatments affected not only the total amount of HSV proteins produced in drug-treated cells, but also

in particular cases, their post-translational processing (Dargan *et al.*, 1986). Both CCX and CBX impaired sulphation, glycosylation and phosphorylation of HSV proteins (although only a small number of HSV phosphoproteins were affected) (Dargan and Subak-Sharp, 1986).

The second effect induced by CCX treatment is a reduction in the numbers of virus particles assembled in drug-treated cell cultures. CCX treatment results in a decrease in protein synthesis *per se* and thus must be a contributing factor in limiting the number of virus particles produced. While, CCX treatment impaired the synthesis of the VSV secondary mRNA transcripts, the mechanism by which this was achieved is not understood. It could involve inhibition of the transcription complex, mobilisation of the cellular stress response, activation of RNases, or modification of cellular transcription factors (Dargan *et al.*, 1992).

Drug-induced impairment of the intracellular transport of virus proteins could also contribute to the reduction in the number of progeny virus particles assembled. In HSV infected BHK cells treated with CCX the relative amounts of particular proteins increased in the nucleus, while decreasing in the cytoplasm, indicating an effect on the nuclear/cytoplasmic transport of these proteins (Dargan and Subak-Sharpe, 1986). Furthermore, treatment of VSV-infected cells with CCX resulted in the accumulation of the virus glycoprotein (G) and matrix protein (M) within the cell (Dargan *et al.*, 1992a). Treatment of VSV-infected cells with the glycosylation inhibitor monensin, also resulted in accumulation of G and M, implicating involvement of the Golgi apparatus. Thus impairment of the assembly of VSV in infected CCX-treated cells is partly due to the inhibition of the transport of the G glycoprotein through the Golgi apparatus to the plasma

membrane, and partly due to a reduction in the amount of virus proteins made in the infected cells (Dargan *et al.*, 1992).

The third effect of triterpenoid compounds is due to virucidal activity. The hydrophilic nature of the triterpenoid compounds and their affinity for cellular membrane, is thought to result in insertion of drug molecules into the virion envelope and/or plasma membranes. Presence of the drug molecules at either of these sites could inhibit virus infection at its earliest stage - virus adsorption or penetration (Dargan *et al.*, 1992b). Pompei *et al.*, (1980) have reported a reduction in HSV-1 infectivity of 100,000-fold, when particles were treated with 8mM GL. The virucidal effect of CCX against HSV-1, HSV-2, and VSV was quite modest at about 10-fold (Dargan *et al.*, 1985 and 1992), but this represents the greatest virucidal activity found for CCX. Only a 2-fold reduction in Ad-5 infectivity resulted from CCX virucidal activity, and no CCX virucidal activity was detected against Reo-3 (Dargan *et al.*, 1992). As Ad-5 is a non-enveloped virus, virucidal activity against this virus is presumably due to non-specific binding of CCX to the surface proteins of the virus particle.

FIGURE 5. Structure of triterpenoid compound.

1.2. RETROVIRUSES.

The retrovirus class of RNA viruses, have received much recent attention due to the threatening HIV epidemic, and the emergence of AIDS as a major threat to human health world-wide. Retroviruses cause a variety of infections, ranging in severity from benign (endogenous retroviruses) to lethal infections (HIV and HTLV-1).

Based on the pathogenesis of the infection by a retrovirus, they have been classified into three groups (Tachibana, 1982, 1985). (1) Oncovirinae: the viruses belonging to this group can induce tumours upon infection. They are also the only group of

exogenous oncoviruses carrying cellular and proto-oncogenes. (2) Leukemovirinae: when expressed can result in rapid leukaemia in experimental animals. (3) Lenti- leukaemia virus and those which induce leukaemia in experimental animals. (4) Lentivirinae: The infection of these viruses leads to the

emergence of a slowly developing disease in experimental animals, eg. caprine arthritis- encephalitis (sheep), equine infectious anaemia (horses), human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS).

in this group. (3) Spumavirinae: The viruses belonging to this group are generally not associated with any known disease in their host (eg. simian foamy virus S).

Retroviruses are enveloped viruses ranging in size from 80 to 120 nm. The retrovirus genome is composed of two identical copies of single-stranded RNA. The

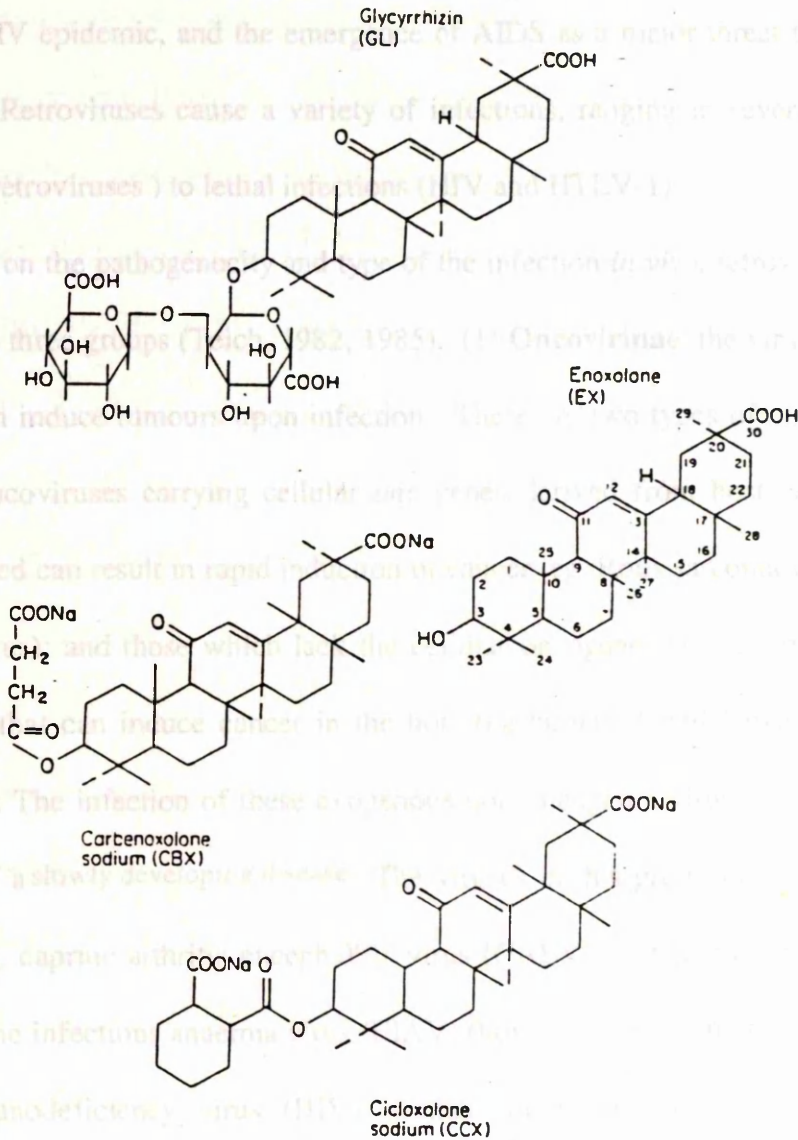


FIGURE 5. Structure of triterpenoid compounds.

1.2. RETROVIRUSES.

The retrovirus class of RNA viruses, have received much recent attention due to the threatening HIV epidemic, and the emergence of AIDS as a major threat to human health world-wide. Retroviruses cause a variety of infections, ranging in severity from benign (endogenous retroviruses) to lethal infections (HIV and HTLV-1).

Based on the pathogenicity and type of the infection *in vivo*, retroviruses have been classified into three groups (Teich, 1982, 1985). (1) **Oncovirinae**: the viruses belonging to this group can induce tumours upon infection. There are two types of oncoviruses: those exogenous oncoviruses carrying cellular *onc* genes derived from host cell DNA, which when expressed can result in rapid induction of cancer (eg. Rous sarcoma virus and Murine leukaemia virus); and those which lack the cellular oncogene, but nevertheless express a viral protein that can induce cancer in the host (eg human T-cell leukaemia virus). (2) **Lentivirinae**: The infection of these exogenous non-oncogenic viruses is characterised by emergence of a slowly developing disease. The viruses in this group mainly infect ungulate mammals. eg. caprine arthritis encephalitis virus (CAEV) (goat), visna maedi virus (VV) (sheep), equine infectious anaemia virus (EIAV) (horses). The immunodeficiency viruses: human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), and bovine immunodeficiency virus (BIV) are also included in this group. (3) **Spumavirinae**: These are viruses also known as foamy viruses, they are generally not associated with any known disease, however, they may cause persistent infection in their host (eg simian foamy virus 5).

Retroviruses are enveloped viruses ranging in size from 100 to 120nm. The retrovirus genome is composed of two subunits of single stranded RNA (of identical

sequence), and ranging in size from 7Kb to 10Kb. All retrovirus genomes are capped at the 5' end and polyadenylated at the 3' end. Other small RNA molecules are associated with the genome, mainly tRNA and small ribosomal RNA s. The t-RNA serves as primer for the synthesis of provirus DNA by viral reverse transcriptase. The retrovirus genome is flanked by two long terminal repeats (LTR s). The LTR s contain many non-coding sequences (from 5' end these are; R, U5, primary binding site (PB), leader, polyurine tract, U3, R, and polyadenylation site).

The first large open reading frame (ORF), adjacent to the 5' LTR, codes for the GAG gene. This gene is translated from full-length RNA, and yields a precursor polyprotein that is cleaved to yield three proteins; matrix (MA), capsid (CA), and nucleocapsid protein (NC). The NC protein is associated with the RNA genome of the mature virus particle. A known DNA binding sequence (CYS-X₂-CYS-X₄-HIS-X₄-CYS) is well conserved among retrovirus nucleocapsid proteins. The MA is a myristoylated membrane associated protein, located between the capsid and the envelope component of the virion. The CA protein is hydrophobic and forms the core structure of the virion.

The second largest ORF in retrovirus genome is pol. The pol ORF generally overlaps that of the gag ORF, but is transcribed from a different reading frame. The pol ORF codes for three proteins; protease (PR), reverse transcriptase/ribonuclease (RT), and integrase/endonuclease (IN). The protease gene is located at the 5' end of the pol ORF and is cleaved from the pol gene polyprotein by an autocatalytic process. The RT consists of a heterodimer of a 66K polypeptide and a 51K polypeptide. The reverse transcriptase of retroviruses specify both RNA-dependent DNA polymerase, and ribonuclease H activities. The reverse transcriptase activities of most retroviruses requires the presence of Mg²⁺

(with the exception of mammalian C-type viruses which prefer Mn^{2+}). The RT region of the pol ORF is one of the most conserved regions⁵ of the retrovirus genome. The integrase gene is located at the carboxy-terminal of the pol ORF and its protein product is responsible for the specific integration of the provirus DNA into the cellular genome. Integration of provirus into cellular DNA sequences occurs at random sites within the host genome. However, on the virus side the integration is specific, occurring at the specific virus sequence. Responds to tat (TAR) is a 59 nucleotide stem loop structure located at the 5'

The third large ORF in retrovirus genome is known as env. The env ORF is the least conserved of all retrovirus genomic sequences. This region encodes for both a surface and a transmembrane protein, which are usually held together in a complex by disulphide bonds, and/or non-covalent interactions. In all retroviruses, the surface protein is heavily glycosylated, and is responsible for the specific binding of the virion to the host cell membrane during virus adsorption. The surface protein is the main target for neutralising antibodies in the infected host. The transmembrane protein has 3 distinct domains; 1. the external domain, which is responsible for attachment to the surface protein, 2. the hydrophobic membrane domain which is responsible for the fusion of the virus envelope with the cell membrane (facilitating virus penetration), and 3. the cytoplasmic domain.

In addition to the three large ORF's, some retroviruses contain additional short ORF's which are important for virus replication. In lentiviruses, and particularly HIV, at least six additional genes have been identified, these are: vif, vpu, vpr, tat, rev and nef (Figure 6). HIV-1 mRNA species Rev does not have any

The HIV-1 tat gene encodes a 86 amino acid protein which is the product of a doubly spliced m-RNA coded for by two ORF's, the first 5' to env and the second

overlapping the transmembrane protein (gp41), but in a different reading frame. The tat protein is mainly localised in the nucleus of infected cells (Sheridan *et al.*, 1993). Tat operates as a trans-activator (Siegel *et al.*, 1986), and has a positive regulatory function (Arya *et al.*, 1987). It is thought that tat increases the rate of transcription initiation, the efficiency of transcript elongation and the stability of the transcription initiation complex (Laspia *et al.*, 1989; reviewed by Cullen, 1990; and Feinberg *et al.*, 1991). The cis-acting sequence that responds to tat (TAR) is a 59 nucleotide stem loop structure located at the 5' end of all HIV-1 transcripts (Muesing *et al.*, 1987). The tat protein operates by specifically binding to TAR element and increasing the efficiency of the RNA polymerase-II mediated transcript elongation process (Marciniak *et al.*, 1991). At least two functional domains of the tat protein have been identified; the first is a highly conserved motif, containing seven cysteine residues, which may mediate protein-protein interactions *in vivo* (Frankel and Pabo., 1988), and the second, rich in lysine and arginine residues, is located at the C-terminal, and is required for the nuclear localisation of the tat protein.

The 116 amino acid protein product of the HIV-1 rev gene arises from a doubly spliced mRNA that overlaps the tat exon (reviewed by Green and Cullen, 1990). The rev protein is located in the nucleus (Cullen *et al.*, 1988), particularly in the nucleolus, of the HIV-infected cells. The rev protein functions as a negative regulator of its own synthesis and also mediates the establishment of an equilibrium between virus structural and regulatory protein synthesis. Rev is required for the cytoplasmic transport of unspliced and singly spliced HIV-1 mRNA species. Rev does not have any effect on the splicing pattern of HIV-mRNAs (Emerman *et al.*, 1989). In the absence of rev, high levels of unspliced transcripts can be detected in the nucleus. Rev has no effect on the cellular location of

spliced or unspliced cellular RNA's. A highly structured 234 nucleotide cis-acting element [rev response element (RRE)], which is important for modulating the level of free rev protein in the nucleus is located within the env gene (Hadzopoulos-cladaras *et al.*, 1989). The binding of rev protein to RRE is essential for the nuclear transport of unspliced and singly spliced mRNA's to the cytoplasm, by directly facilitating their interaction with a component of the cellular RNA transport pathway (Malim *et al.*, 1988).

The HIV-1 nef gene overlaps the 3' LTR and encodes a 27Kd protein, which is translated from a multiply spliced mRNA transcript (Harris *et al.*, 1992). Nef-minus mutants replicate at 2 to 10 fold higher titre than the wild type virus (Luciw *et al.*, 1987), demonstrating the negative regulatory role of this protein. Additional functions attributed to nef include; involvement in signal transduction pathways (Guy *et al.*, 1987), and regulation of CD4 receptor expression (Guy *et al.*, 1987; Garcia and Miller, 1991). Other workers, however, (Cheng-Mayer *et al.*, 1989; Bachalerie *et al.*, 1990; Harris *et al.*, 1992) have failed to detect any of these additional functions attributed to nef.

The HIV-1 vif gene encodes a 23 Kd protein (Kan *et al.*, 1986; Lee *et al.*, 1986), which plays a role in virus infectivity (Strebel *et al.*, 1987). Vif-minus mutants, produced wild type level viral mRNA, proteins, and virus particles, but the cell-free virions produced were not infectious (Fisher *et al.*, 1987).

The HIV-1 vpr gene encodes a 78 amino acid membrane-associated protein (Wong *et al.*, 1987; Sato *et al.*, 1990), which appears to act as a weak transcriptional activator.

The HIV-1 vpu gene encodes an 81 amino acid protein, which is thought to play a role in virus maturation (Strebel *et al.*, 1988). Infection with vpu minus mutants results in impairment of the release of the virus and accumulation of virion proteins (Strebel *et al.*,

1989; Terwilliger *et al.*, 1989; Klimkait *et al.*, 1990). Interestingly the HIV-1 vpu gene is absent in closely related viruses; HIV-2 and SIV. In contrast, HIV-2 and SIV contain a gene known as vpx, which is not present in HIV-1. The vpx gene encodes a 14 Kd protein, which is abundant in cells infected with HIV-2 or SIV (Henderson *et al.*, 1988). It has been suggested that vpx might have role in cell tropism of the virus (Guyader *et al.*, 1989).

REVERSE TRANSCRIPTION

REPLICATION OF RETROVIRUSES

Retroviruses can be classified as simple or complex types, based on their genomic organisation and regulation of their genome expression in the infected cell (Cullen *et al.*, 1993). There are several criteria that distinguishes complex retroviruses (HIV or FIV) from simple retroviruses (FeLV-A or MLV); complex retroviruses encode multiply-spliced mRNA's, that give rise to proteins with regulatory functions (eg tat and rev).

ATTACHMENT AND VIRUS ENTRY.

Retrovirus infection initiates with the binding of virus surface glycoprotein to a specific receptor on the cell surface. A hypervariable region (V3.3) in the HIV-1 surface protein is required for efficient virus entry (Clements *et al.*, 1991). The virus then enters the host cell by the mechanism of membrane fusion (Marsh and Helenius, 1989). In the case of HIV-1 this process is mediated by the amino-terminus domain of the transmembrane protein (gp41) (Kowalski *et al.*, 1987; Freed *et al.*, 1990). With the exception of ectropic murine leukaemia virus (MLV-E), retrovirus entry into the host cell is a pH-independent process (McClure *et al.*, 1988 and 1990).

UNCOATING.

During the entry of the virus into the cell, virus envelope is lost, the capsid structure disintegrates, and the functional RNA genome is released into the cytoplasm of the infected cell. Little is known of the precise details of retrovirus uncoating.

REVERSE TRANSCRIPTION.

During reverse transcription the virus RT transcribes the single-stranded virus RNA genome into a double-stranded DNA sequence (known as the provirus). The RNase H activity of RT specifically degrades the RNA template from the RNA-DNA hybrid to permit synthesis of the double stranded provirus DNA. During the process of reverse transcription the RT enzyme switches several times from one strand to another strand at random, a mechanism which increases the frequency of genetic recombination events (Hu and Temin, 1990).

INTEGRATION OF THE PROVIRUS.

Following synthesis and migration of the provirus to the nucleus, the next step in retroviral replication is integration of the provirus into the host cell genome. Based on the studies on the retrovirus; murine leukaemia virus. The integrase protein and the two inverted LTR's at the end of the provirus DNA are required for the process of integration (Colicelli and Goff, 1985). In general, integration of the provirus, is an essential step in productive retrovirus replication, however, avian spleen necrosis virus integrase-minus mutants were able to produce infectious virus in infected cells (Panganiban *et al.*, 1983). In general unintegrated provirus DNA can be detected in the cytoplasm of retrovirus-infected

cells, including HIV-1 infected cells. Stevenson *et al.*, (1990) have reported that HIV antigens were present in the supernatant of lymphocyte cell cultures, which have been infected with HIV-1 strains deficient in integrase; however, no infectious virus was isolated from these cultures. These results indicate that unintegrated provirus DNA can serve as the template for HIV-1 core and envelope protein production. Accumulation of unintegrated provirus DNA *in vivo* in HIV-1 infected cells may play a role in the cytopathic effect of the virus.

TRANSCRIPTION AND TRANSLATION OF THE VIRAL PROTEINS.

Transcription of the provirus is carried out by cellular RNA polymerase II. In simple and complex type retroviruses, expression of provirus DNA is controlled by host cell transcription factors. However, in complex type retroviruses, such as HIV-1, the virus LTR sequences contain several cis-acting recognition sites for host cell transcription factors which allows a more sophisticated control of viral gene expression. In all retroviruses, transcription results in an unspliced (gag and pol), or singly spliced (env) mRNA. However, the genomes of complex type retroviruses, such as HIV or FIV, encode shorter multiply-spliced mRNA that regulate virus gene expression. (Cullen *et al.*, 1991). Expression of multiply-spliced viral mRNA's occurs early (Kim *et al.*, 1989), whereas unspliced or singly-spliced mRNA's which encode viral structural proteins are expressed later in the virus replication cycle (Feinberg *et al.*, 1986; Felber *et al.*, 1990). In contrast in simple type retroviruses the availability of cellular transcription factors is responsible for the control of provirus expression and the pattern of mRNA processing.

VIRION ASSEMBLY AND RELEASE

The assembly of retrovirus particles and their release from infected cells is thought to be similar for both the complex and simple type retroviruses (Cullen *et al.*, 1993). Virus assembly takes place at a location adjacent to the plasma membrane of the infected cell. In the case of HIV-1, the myristoylated N-terminal of both the gag and gag-pol polyproteins plays an important role in the assembly of the virion (Gottlinger *et al.*, 1989). The interaction between the zinc-finger motif of the HIV-1 nucleocapsid protein (Aldovini and Young, 1990) and a signal, in the leader sequence, of genomic RNA is important for the packaging of two subunits of genomic RNA within the budding virion. All retroviruses bud through the plasma membrane, which is abundant in viral glycoproteins. Thus the viral envelope acquired during the budding process exhibits glycoprotein spikes projected on the surface. Either, during or soon after, release of the virus from the cell surface, the gag and gag-pol precursor proteins are cleaved by the action of the virus protease to render the virion infectious (Skala *et al.*, 1989; Kohl *et al.*, 1988)

1.2.1. FELINE IMMUNODEFFICIENCY VIRUS (FIV).

FIV is a T-lymphotropic lentivirus originally isolated from a group of cats with an immunodeficiency -like syndrome (Pederson *et al.*, 1987). FIV has proved to be a valuable animal model for human lentivirus infection, and has facilitated understanding of the pathogenesis of HIV-induced AIDS. Furthermore FIV provides an interesting and potentially useful model for the development of lentivirus antiviral and vaccine strategies. It is thought that FIV infection of cats has a long history (Sodora *et al.*, 1994). The prevalence of FIV infection in domestic cats ranges from 1% (central Europe and USA) to 30% (Japan and Australia). The rate of infection among male cats is two, or more, times higher than among female cats, and is usually acquired after the first year (Bendinelli *et al.*, 1995). Viruses similar to FIV may infect large cat wild life populations, including; cougars of Northern America, cheetahs of the Sergenti ecosystem (Olmsted *et al.*, 1992), and lions (Brown *et al.*, 1994). No disease is associated with the lion lentivirus (FIV-Ple) (Brown *et al.*, 1994). Although the mode of FIV transmission among domestic cats has not yet been precisely defined, there is some evidence to suggest that biting is the predominant route of transmission (Bendinelli, *et al.*, 1995). The presence of FIV in the saliva of experimentally and naturally infected cats supports this view (Matteucci *et al.*, 1993). Prenatal and postnatal routes of vertical transmission of FIV, have been reported. Foetal infection, in the uterus, leads to abnormalities in the foetus including; arrested foetal development, subnormal birth weight, and T-cell deficiency. Foster-nursing experiments indicate that kittens can be infected via the milk of an infected mother. Vertical transmission of FIV during the acute maternal infection is about 70% (Oneal *et al.*, 1995). Transmission of FIV through sexual contact has never been clearly confirmed (Hosie, *et al.*,

1990), while airborne transmission of FIV is very unlikely. Like HIV, FIV can be transmitted across mucosal surfaces by infected cells. Moench, *et al.*, (1993), have reported that FIV-infected T-cells are capable of transmitting FIV infection through the mucosal surfaces of the vagina, rectum, or oral cavity. Neutralising maternal antibodies from naturally infected, or vaccinated, cats protect kittens from FIV infection (Pu *et al.*, 1995).

FIV infection *in vivo* initiates most likely in lymphoid tissue. One week after infection, FIV DNA and/or RNA can be detected in bone marrow, thymus, and lymph nodes. The salivary gland was the only nonlymphoid tissue in which FIV was detected (Matteucci *et al.*, 1993). In the later stage of infection (3 weeks post-infection), most of the nonlymphoid tissues examined, were positive for the presence FIV. The presence of anti-FIV antibodies in the serum of experimentally infected cats could be detected within 2 to 6 weeks post-infection. Most of the antibodies produced early in the infection, are directed toward the virus glycoproteins and/or the major core proteins (Lombardi *et al.*, 1993). The presence of neutralising antibodies can be detected 5 to 6 weeks post-infection and the titres usually remain high throughout life, reaching a plateau after 10 to 11 months post-infection (Tozzini *et al.*, 1992). Sera isolated in the field from different cats, neutralised different FIV isolates indicating that FIV, like HIV, has antigenic epitopes capable of inducing broad-range neutralising antibodies (Tozzini *et al.*, 1992). One such epitope maps to the V3 region of the FIV surface protein (Lombardi *et al.*, 1993).

Different isolates of FIV differed in their ability to infect different cell lines in *in vitro* experiments, having the same surface phenotype (Phillips *et al.*, 1990). Differences in the lymph nodes. During this period, some clinical symptoms appear such as: anorexia, virus env proteins are thought to account for these differences in cell tropism. It has been unknown recurrent fevers, anorexia, weight loss and apparent changes in sleep patterns.

shown that virus cell tropism can be affected by the passage history of the virus, possibly due to the different host cell membrane proteins acquired by the virus during the budding process, however, the mechanism by which this operates is unclear (Orentas *et al.*, 1993). Appearance of FIV cytopathic effect in different cell lines may depend on the FIV isolate/host cell system used (Tokunaga *et al.*, 1992). Dow *et al.*, (1992) have reported that, among neuronal cells susceptible to FIV infection, astrocytes were the most sensitive (producing syncytium and cell death), whereas microglial cells yielded infectious virus in the absence of any CPE, while endothelial cells gave no apparent CPE, and little virus yield.

MORP The Clinical manifestations of *in vivo* FIV infection are very similar to that of HIV infection of humans. FIV infections in cats can be divided in 5 different phases based on the appearance and severity of the clinical symptom; (1) Acute phase, which is a transient stage following the primary infection and is accompanied by a range of clinical symptoms (Lymphadenopathy, acute diarrhoea, conjunctivitis, dermatitis, gingivitis, and mild upper respiratory infections (Beebe *et al.*, 1994; Torten *et al.*, 1991); (2) Asymptomatic phase, which is characterised by the presence of an occult and clinically inapparent infection over a long period of time, during which haematological and immunological abnormalities due to the infection of FIV are detectable (Ackley *et al.*, 1990; Matteucci *et al.*, 1993). During this period virus can be isolated from the plasma and saliva of the asymptomatic cats with the same frequency as the diseased cats (Matteucci *et al.*, 1993); (3) persistent generalised lymphadenopathy, which is characterised by the persistent activation and enlargement of the lymph nodes. During this period, some clinical symptoms appear such as, etiologically unknown recurrent fevers, anorexia, weight loss and apparent changes in sleeping patterns

(reviewed by Bendinelli *et al.*, 1995); (4) ARC (AIDS-related-complexes), a range of chronic secondary upper-respiratory and oral infections caused by other micro-organisms appears in infected cats during this period. Other symptoms such as neurological abnormalities, alopecia, and neoplasia may also occur (Reviewed by Bendinelli *et al.*, 1995); (5) FAIDS (feline immunodeficiency syndrome), this period is marked by the appearance of a variety of opportunistic infections, neoplasia, severe anaemia, leukopenia, and neurological disorders, most cats die within a year of the diagnosis of FAIDS (Bendinelli *et al.*, 1995).

MORPHOLOGY AND GENOMIC STRUCTURE OF THE VIRION.

The mature FIV particle consists of a capsid, surrounded by an outer envelope membrane derived from the host cell plasma membrane. The particle is 100 to 125nm in diameter and has spikes containing viral glycoproteins on the surface of the virus envelope (Pederson, *et al.* 1987).

The virus genome consists of two copies of positive-stranded polyadenylated RNA. Like other lentiviruses, FIV has a complex genomic structure. The genome encodes three large open reading frames corresponding to GAG, POL and ENV, flanked by two LTRs (variously reported to be 355 to 361 base pair long) which are bordered by two base pair inverted repeats (Talbot, *et al.* 1989; Maki, *et al.*, 1992) (Figure 6). In addition, the genome contains at least four short open reading frames (Olmsted, *et al.* 1989), three of which (Vif, ORF A, and Rev) have been shown to encode protein products (Miyazawa, *et al.* 1994).

The FIV LTR element contains three regions; the U3 region (216 base pairs), the R region (65 base pairs), and the U5 region (65-68 base pairs) (Olmsted, *et al.* 1989; Talbott, *et al.*, 1989). FIV LTRs appear to be strong basal promoters, at least in some cell types (Miyazawa, *et al.*, 1992 and Sparger, *et al.*, 1992). The regulatory cis-acting sequences detected in FIV LTRs include a consensus polyadenylation signal in the R region, two TATA like sequences, a CCATT sequence, and several transcription factor binding sites (AP-1, Ap-4, ATF-1, EBP-20, and NF- κ B) in the U region (Kawaguchi, *et al.*, 1991 and 1992, Sparger, *et al.*, 1992, and Miyazawa, *et al.*, 1994). The AP-1 and Ap-4 binding sites are important for basal promoter activity in non-lymphoid feline cell lines (Kawaguchi, *et al.*, 1992, and Sparger, *et al.*, 1992), however the presence of an AP-1 binding site is not required for FIV replication in feline T lymphocytes (Miyazawa, *et al.*, 1993). The presence of a stem-loop structure, reminiscent of the HIV-1 TAR element has been reported in the R region of the FIV LTR, but at the present time there is no evidence for protein binding to this structure (Miyazawa, *et al.*, 1991; Morikawa, *et al.*, 1991). The presence of a negative regulatory element (NRE) similar to that present in HIV (Siekevitz, *et al.*, 1987), has been located within the first 62 bases of the LTR of the Petaluma strain of FIV (Sparger, *et al.*, 1992). However, a NRE sequence was not detected in another strain of FIV (FIV TM1 (Kawaguchi, *et al.*, 1992).

Six different classes of viral specific m-RNA were identified in FIV infected cells; 9.2 Kb, 5.2 Kb, 4.4Kb, (doublet), 1.7 Kb, and 1.4 kb (Tomonaga, *et al.*, 1993). The 9.2 Kb, 5.2 Kb, and 4.4 Kb (doublet mRNAs) are not expressed in the cytoplasm of cells transfected with rev minus mutants (Tomonaga, *et al.*, 1993). The region between the POL and ENV genes contains at least three splice acceptor and two splice donor sites. The 9.2

kb mRNA is considered to be the primary transcript for the GAG-POL precursor polyprotein, the Vif and env mRNAs (5.2 and 4.4 kb respectively) are formed by a single splicing event, and the Rev mRNA arises from multiple splicing of the primary transcript (Tomonaga, *et al.*, 1993a). The mRNA for the ORF A (putative tat gene) seems to contain the entire env and rev genes (4.4kb) or two rev-coding exons (1.7kb) (Miyazawa *et al.*, 1994). The FIV virus core structure is derived from the GAG-POL polyprotein precursor. The GAG gene of FIV encodes a 450 amino acid precursor polyprotein of about 50k (Maki, *et al.*, 1992), which is post-translationally cleaved to yield three protein products; the matrix (p15), the capsid (p24), and the nucleocapsid (p10) proteins (Egberink, *et al.*, 1990a; Steinman, *et al.*, 1990). Serological cross reactivity observed to the FIV capsid (CA) protein among FIV, CAEV, and visna virus, indicates the presence of a conserved region within this protein (Olmsted, *et al.*, 1989; Egberink, *et al.*, 1990). The FIV matrix protein is myristoylated and starts with a Met-Gly-Asn-Gly sequence (Elder, *et al.*, 1993), which is identical to the myristoylation signal of the HIV matrix protein (Talbot, *et al.*, 1989). Interestingly the matrix proteins of other non-primate lentiviruses (EIAV and visna virus) are not myristoylated (Miyazawa, *et al.*, 1994). The FIV nucleocapsid protein (p10) contains two sequences of Cys-(X)₂-Cys (X)₄-His-(X)₄-Cys (Maki, *et al.*, 1992), which are characteristic of retroviral nucleic acid binding proteins (Gorelic, *et al.*, 1988).

The FIV POL gene which encodes the polymerase gene products (bases 1868-5239) is the second largest ORF and overlaps the GAG gene by 109 base pairs (Talbot *et al.*, 1989, and Olmsted *et al.*, 1989). The POL gene is in a -1 reading frame with respect to the GAG gene and a GAG-POL polyprotein is synthesised by ribosomal frame shifting as in the case of primate lentiviruses (Olmsted *et al.*, 1989; Morikawa and Bishop, 1991; Miyazawa

et al., 1994). The overlapping region contains the GGGAAAC sequence required for ribosomal frame shifting. The GAG-POL polyprotein is cleaved to generate the protease (p13), reverse transcriptase (p61), integrase (p14), dUTPase (p14). FIV reverse transcriptase is very similar to that of HIV (more than 45% homology in genome sequence) (Talbot, *et al.*, 1989). FIV RT is a Mg^{2+} dependent enzyme with dual enzymatic functions, having both DNA polymerase and RNase H activities (Cronn, *et al.*, 1992). dUTPase is not encoded by primate lentiviruses, but is specified by EIAV, visna virus and CAEV (Miyazawa *et al.*, 1994). The FIV dUTPase (p14) protects against misincorporation of deoxyuridine into viral DNA, thus increasing the fidelity of genome replication (Elder *et al.*, 1992; Lerner *et al.*, 1995). While dUTPase activity is not essential for FIV replication, it may be required for the replication of FIV in non-dividing cells such as non-dividing macrophages, or resting T lymphocytes (Miyazawa, *et al.*, 1994). Indeed, dUTPase activity is required for the replication of EIVA in non-dividing equine macrophages (Threadgill *et al.*, 1993).

The env gene is encoded by the third largest ORF in the FIV genome (bases 6250-8850), and overlaps the 3' end of open reading frame C. The env gene encodes a precursor glycoprotein (gp160) which is trimmed soon after synthesis, before the protein is transported from the endoplasmic reticulum yielding a glycoprotein of 130K Mwt (gp130). Gp 130 is further cleaved during passage through the Golgi apparatus into the mature gp 95 (surface protein), and gp41 (transmembrane) glycoproteins (Stephens *et al.*, 1991). The env gene nucleic acid sequence is the least conserved sequence among FIV isolates. The FIV env gene exhibits 9 variable and hypervariable regions (V1-V9); of which V1 and V2 are located in the leader sequence of the env gene; four (V3-V6) are located in the sequences

coding for surface protein; and 3 (V7-V9) are located in the transmembrane protein env regions (Pancino *et al.*, 1993 and 1993a). The sequence variability observed in the V3 and V4 regions of FIV surface protein is analogous to that found for the V3 loop of the HIV-1 gp120 surface protein (Pancino, *et al.*, 1993). Lombardi, *et al.* (1993) have mapped a linear epitope for the binding of B-cells to the third variable region (V3) of the FIV (gp95) surface protein. The presence of a neutralising domain in the hypervariable region of HIV-1 and HIV-2 envelope glycoproteins is well established (Wolf, *et al.*, 1993). The FIV surface protein V3 region also contains at least 4 binding sites for neutralising antibodies. Two of the FIV epitopes for neutralising antibodies are positioned towards the amino-terminal and two towards the carboxy terminal of the V3 region (Lombardi, *et al.*, 1995). The two surface glycoprotein epitopes, which mapped to the V3 carboxy terminal are detectable on the surface of mature FIV particle and in the cytoplasm of FIV infected cells, but are not detectable when the protein is expressed on the surface of the plasma membrane of infected cells. Lombardi *et al.* (1995) demonstrated that the epitopes involved in binding neutralising antibodies are discontinuous.

Greene, *et al.* (1993) have reported that, in response to pressure from the host immune system, the FIV env gene has a 3×10^{-3} nucleotide substitution rate. FIV isolates are grouped in at least 3 distinct subtypes depending upon the nucleotide sequence of the env gene (Sordora *et al.*, 1994). Different world-wide isolates of FIV have been classified into four major subtypes A, B, C, and D, based on phylogenetic analysis of the V3 and V5 regions in the FIV env surface protein. Among the different subtypes, sequence divergence ranged from 18% to 26%. Within each subtype, the sequence divergence varied by 3% to

15%. The env gene nucleotide sequence from individual infected cats varied by as much as 4% (Sodora, *et al.*, 1994).

The FIV regulatory vif gene (virus infectivity factor) overlaps the 3' end of pol gene and is similar in size and location to the vif genes of primate lentiviruses (Talbot *et al.*, 1989, and Philips *et al.*, 1990). The FIV vif gene product is hydrophilic and basic (Maki *et al.*, 1992). FIV vif-minus mutants transfected into CrFK cells produced wild type level of reverse transcriptase, but infection was only possible by cocultivation with FIV-infected cells, and not with cell-free virus (Tomonaga *et al.*, 1992). Indeed, virus isolated from the vif-minus transfected cells failed to infect feline CD4 positive T cells (Tomonaga, *et al.*, 1992). Furthermore, Shacklett, and Luciw, (1994) have shown that transfection of CrFK cells with FIV proviruses containing deletion in different regions of the vif gene yielded very little cell-free virus or p24 gag viral protein. Therefore it is concluded that the vif gene is important for FIV infectivity in CrFK cells *in vivo*.

The FIV Rev gene consists of two exons; the first (ORF B) located at an intergenic region (at the 3' end of the Pol gene through the L region of the env gene and utilises the first 81 amino acids of the env precursor) (Kiyomasu *et al.*, 1991; Philips *et al.*, 1990; Tomonaga *et al.*, 1993). The second exon (ORF H) is located immediately downstream from the env gene and overlaps the LTR (Kiyomasu, *et al.*, 1991; Philips, *et al.*, 1992). The FIV Rev response element (RRE) is located at the 3' end of the env ORF and partially overlaps the 5' end of LTR (Philips, *et al.*, 1992), while those of the primate lentiviruses are located entirely within the env gene (Rosen, *et al.*, 1988; Malim, *et al.*, 1989). In the absence of rev, no unspliced or singly spliced FIV mRNA is expressed, and virus

production is inhibited (Kiyomasu, *et al.*, 1991; Tomonaga, *et al.*, 1993), thus Rev seems to be essential for FIV replication.

The putative FIV TAT gene (ORF A) is located between the *vif* and the *rev* genes and is similar in size and location to the first exon of primate lentivirus *tat* genes. However, ORF A is not essential for FIV replication in feline T lymphoblastoid cell lines, and some strains of FIV do not have detectable *tat* activity (Miyazawa, *et al.*, 1993; Tomonaga, *et al.*, 1993). Nevertheless, ORF A-minus mutants replicate poorly in primary peripheral blood lymphocytes (Tomonaga, *et al.*, 1993a), suggesting that ORF A may have a role in FIV replication *in vivo*.

FIV cell tropism in infected cats is analogous to that of HIV in humans, in that the main target is the T lymphocyte. However, FIV also infects monocytes, macrophages, microglial cells, and astrocytes, as does HIV (Dow, *et al.*, 1992; Brunner and Pederson, 1989). Early in infection, and before the onset of the clinical manifestations of the disease, between 25% to 75% of infected cells are T-lymphocytes, with a small percentage of macrophages (2%-10%) also infected. Beebe, *et al.*, (1994) have reported that in the later stages of infection (after the appearance of the clinical signs) the percentage of macrophages which are infected increased dramatically (40%-90%), and have suggested that, as FIV infection is less cytopathic in macrophages than T-lymphocytes, the shift from T-cell infection to macrophages late in the FIV infection could be important for viral persistence. Although, the CD4⁺ lymphocytes decline is observed in FIV-infected cats (Hofmann-Fezer *et al.*, 1992), it has been demonstrated that there is no correlation between the presence of CD4 molecules on the cell surface and the ability of the cell to harbour FIV

(Brown, *et al.*, 1991). Recently Hosie *et al.*, (1993) have isolated a monoclonal anti-idiotype antibody (designated vpg15), which can block FIV infection of susceptible cat cells, by binding to a specific receptor on the cell surface, thus blocking virus adsorption. This antibody identified a 24-kd protein on the surface of susceptible cells. This protein has been identified as the feline homologue of human CD9 (Willet, *et al.*, 1994).

FeLV particles are about 100nm in diameter and are composed of an icosahedral capsid surrounded by envelope derived from the plasma membrane of the infected cell. The viral genome consists of two positive sense linear, single-stranded RNA molecules in the form of a dimer. The genomic RNA is capped at the 5' end and polyadenylated at the 3' end. The FeLV genome consists of ~8kb of nucleotide sequences encoding the three retrovirus large open reading frames: gag, pol, and env (Figure 6).

The FeLV gag gene encodes a 501 amino acid non glycosylated precursor polyprotein (Pr65^{gag}), which is posttranscriptionally cleaved by the virus encoded aspartate protease to yield: P15 (matrix protein), P27 (major capsid protein), P9 (env glycoprotein), and P10 (nucleocapsid protein) (Kim and Stephens, 1987).

The FeLV pol gene encodes a 1708 amino acid precursor polyprotein (Pr180^{pol}), which is cleaved by virus protease into: p15 (matrix protein), p13 (protease), p42 (integrase), and reverse transcriptase (p66) (Kim and Stephens, 1987; 1979; Donahue *et al.*, 1988).

The FeLV env gene encodes a precursor polyprotein (Pr120^{env}) consisting of a 15 amino acid leader sequence, followed by a 412 amino acid gp120 protein (p120) and a 68 amino acid transmembrane protein (p15E) (Neil *et al.*, 1980; Donahue *et al.*, 1988).

1.2.2. FELINE LEUKAEMIA VIRUS (FELV).

Feline leukaemia virus is a class C-retroviruses, which has been associated with immunodeficient diseases, malignancy, and degenerative diseases of haematopoietic origin (Essex, 1975). Indeed, feline leukaemia viruses were first isolated from a group of cats with lymphosarcoma (Jarret *et al.*, 1964).

FeLV particles are about 100nm in diameter and are composed of an icosahedral capsid surrounded by envelope derived from the plasma membrane of the infected cells. The viral genome consists of two positive sense linear, single-stranded RNA molecules in the form of a dimer. The genomic RNA is capped at the 5' end and polyadenylated at the 3' end. The FeLV genome consists of ~ 8Kb of nucleotide sequences encoding the three retrovirus large open reading frames; gag, pol, and env (Figure 6).

The FeLV gag gene encodes a 501 amino acid non-glycosylated precursor polyprotein (Pr65^{gag}), which is posttranscriptionally cleaved by the virus encoded aspartyl protease to yield ; P15 (matrix protein), P27 (major capsid protein), P12 (structural protein), and P10 (nucleocapsid protein) (Khan and Stephens, 1977).

The FeLV pol gene encodes a 1,709 amino acid precursor gag-pol polyprotein (Pr180^{gag-pol}), which is cleaved by virus protease during, or after virus budding to yield the protease (p13), integrase (p47), and reverse transcriptase (p70) proteins (Rho and Gallo., 1979; Donahue *et al.*, 1988).

The FeLV env gene encodes a precursor polyprotein (Pr⁸⁵), with a 33 amino acid leader sequence, followed by a 412 amino acid surface protein (gp70), and a 197 amino acid transmembrane protein (p15E) (Neil *et al.*, 1980; Donahue *et al.*, 1988).

In addition to the three major ORF's the FeLV genome also encodes number of potential short, spliced, orfs, but as yet it is not clear whether any of these specify a functional protein (Donahue *et al.*, 1988).

The LTRs of FeLV consist of 480-560 bp sequences which contain promoter and enhancer elements required for viral transcription, and also contain signals for polyadenylation.

FeLV isolates fall into one of three subgroups; A, B, or C, depending upon their behaviour in virus neutralisation and virus superinfection interference assays (Samara and Log, 1973). The behaviour of the viruses in such assays is due to differences in the viral envelope glycoprotein (p70) antigen (Samara and Log, 1973).

All of the cats infected naturally with FeLV have the FeLV-A subgroup virus, either alone or in combination with one or other of the subgroup viruses. Thus, FeLV-B isolates are present along with FeLV-A in approximately 40% of naturally occurring infections, while the FeLV-C subgroup isolates are found in only 1% (Jarret *et al.*, 1978).

The FeLV subgroup viruses differ in host cell range; FeLV-A isolates are generally restricted to growth in feline cells, while FeLV-B and C isolates can grow in cells from a variety of species including; feline, human, and canine cells. The extended host cell range is due to differences in the outer surface glycoprotein (gp70) (Jarret *et al.*, 1973). Although, FeLV (B and C) can grow in human cells in tissue culture, there seems to be little risk involved of FeLV transmission from cats to humans (Hardy *et al.*, 1976).

In a study by Hardy *et al.*, (1976) involving 2000 cats, 12% of the uninfected cats housed along with persistently infected cats became infected with FeLV. Cats persistently infected with FeLV shed virus in the saliva, urine and faeces (Hardy *et al.*, 1973). The

main route of transmission is probably via saliva, perhaps by biting or mutual grooming (Hardy *et al.*, 1973).

While in natural infection, FeLV-A is transmitted horizontally, the B and C subgroup viruses can only be transmitted in the presence of FeLV-A (Jarret *et al.*, 1978).

FeLV-B infections of cats is associated with leukaemia, while FeLV-C infections is associated with aplastic anaemia. Both FeLV-B and FeLV-C are derived by genetic recombination between FeLV-A and endogenous FeLV-like sequences (enFeLV) carried in the genome of domestic cats (Russel and Jarret, 1978; Stewart *et al.*, 1986; Overbaugh *et al.*, 1988). These endogenous FeLV-like sequences cannot be induced to produce infectious virus particles (Benveniste *et al.*, 1975).

The enFeLV sequences (~ 4kbp long) are significantly shorter than the FeLV genome, having 3.3 to 3.6 kb deletions in the gag-pol region and ~ 0.7 to 1.0 kb deletion in the env gene. Both LTR's are however, intact (Soe *et al.*, 1983). EnFeLV sequences are present at multiple locations in feline genome and are arranged in a nontandem fashion. Although, the sites of integration of enFeLV sequences in the host DNA is identical in different tissues from the same animal, these sites vary between different individuals (Koshy *et al.*, 1980).

Recombination between FeLV-A and enFeLV is mostly confined to the env gene; FeLV recombinants having exchanged up to 60% of the surface gp N-terminal sequence have been reported. This substitution probably accounts for the expanded host range of the recombinant isolates (Sheets *et al.*, 1992).

Three forms of clinically distinct FeLV-associated lymphomas have been described. Firstly, a Thymic lymphoma characterised by a rapidly progressive tumour. Secondly,

Alimentary lymphoma, with tumours in the gastrointestinal tract, and finally, multicentric lymphoma, in which many different tissues and lymphoid organs are involved (Cotter *et al.*, 1992).

Although, the mechanism by which FeLV induces tumors is not fully understood, transduction of a host proto-oncogene, and/or insertional mutagenesis of host gene might be involved (Levy *et al.*, 1993).

By altering the regulation of the expression of the host genes the LTR's play an important role in FeLV-related leukaemia. The LTR sequences from the proviruses isolated from cats with a thymic lymphosarcoma have repeated enhancer elements, not present in non-leukomogenic viruses (Fulton *et al.*, 1990). Viruses with multiple enhancer elements can be generated after only a single passage in cats (Rhon and Overbaugh, 1995). It seems possible that the enhancer sequence might play a role in leukemogenesis, through insertional activation of cellular proto-oncogenes. Insertion of a FeLV provirus upstream of the natural promoter of a proto-oncogene resulted in deregulation of the promoter and overexpression of the gene product. Thus, FeLV viruses can activate transcription of cellular proto-oncogenes to a level sufficient for tumour induction (Rhon and Overbaugh, 1995).

FeLV can cause a severe immunosuppression of cats (FeLV-FAIDS). The course of the disease depends on the age of the cat; younger cats (< 8 weeks old) develop acute disease within 6 months of infection, while older cats remain asymptomatic for up to 2 years, but thereafter advance to a full blown immunodeficiency disease reminiscent of human AIDS (Overbaugh *et al.*, 1988a). Defective FeLV virus isolates associated with acute immunodeficiency have been isolated, and have been shown to consist of subgroup A

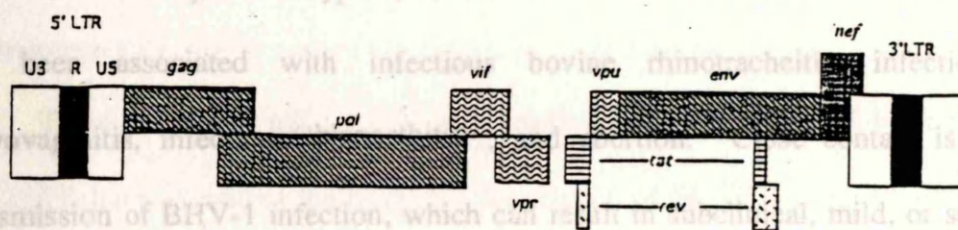
viruses (clone 61E) mixed together with a family of immunodeficiency-disease- associated variants (possibly From subgroup B) (clone 61C). The latter, although replication defective, are propagated in the presence of FeLV-A (61E), which functions as a helper virus (Overbaugh *et al.*, 1988a). An FeLV-FAIDS virus isolated from an infected cat and now regarded as the prototype of FeLV-FAIDS has a 6 amino acid deletion near the amino terminus of the gp 70 and a 6 amino acid insertion near the carboxy terminus, as well as 11 scattered amino acid changes throughout the protein (Overbaugh *et al.*, 1988a). The prototype FeLV-FAIDS virus (61C) also has point mutations at two locations within the LTR U3 region and a nucleotide deletion at the boundary between the U3 and the R region (Overbaugh *et al.*, 1988a). The extensive destruction of cat T cells by 61E/61C isolates is associated with massive superinfection (Donahue *et al.*, 1991).

Although, the majority of cats infected with FeLV develop viraemia they acquire immunity and eventually clear the infection. Cats which harbour latent FeLV-A may remain healthy for up to 3 years before becoming ill with FeLV-related diseases.

FIGURE 6. Schematic representation of HIV, FIV, and FeLV-A genomic organisation.

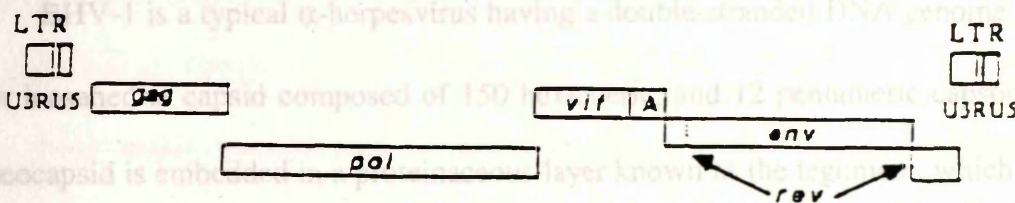
1.3. BOVINE HERPESVIRUS TYPE 1 (BHV-1):

Bovine herpesvirus type-1, also known as infectious bovine rhinotracheitis virus has been associated with infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, and infectious abortion. It is required for transmission of BHV-1 infection, which can result in subclinical, mild, or severe disease. Because BHV-1 infection is of economic importance, several vaccines (mostly live attenuated) have been developed by different agencies to prevent the spread of viral infection.



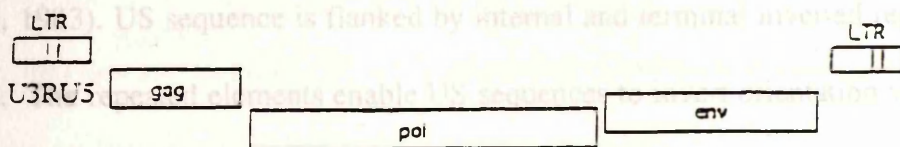
HIV-1

HIV-1 is a typical α -herpesvirus having a double-stranded DNA genome packaged in a nucleocapsid composed of 150 proteins and 12 pentameric subunits. The nucleocapsid is embedded in a lipid bilayer known as the envelope, which is in turn surrounded by a lipid envelope, derived from host cell nuclear membrane.



FIV

The genome of BHV-1 contains 137kb of linear double stranded DNA, organised as, long (UL; 105kb) and short (US; 31kb) segments of unique DNA sequence (Mason et al., 1981). US sequence is flanked by internal and terminal inverted repeats. The 12kb UL segment contains elements enable US sequences to be integrated into the host cell genome, giving two isomeric forms of the BHV-1 genome (Mason et al., 1981).



FeLV-A

As with other herpes viruses, the replication and assembly of FeLV-A occurs in the nucleus of the infected cell. The FeLV-A genome is sequentially transcribed in a cascading manner. These three class of genes are: immediate early (IE), early (E), and late (L). Expression of IE genes does not require the expression of other genes, while expression of the L genes is dependant upon viral DNA synthesis and expression of E

FIGURE 6 . Schematic representation of HIV, FIV, and FeLV-A genomic-organisation.

1.3. **BOVINE HERPESVIRUS TYPE 1 (BHV-1).**

Bovine herpesvirus type-1, also known as infectious bovine rhinotracheitis virus has been associated with infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, infectious balanitis, and abortion. Close contact is required for transmission of BHV-1 infection, which can result in subclinical, mild, or severe disease. Because BHV-1 infection is of economic importance, several of vaccines (mostly live attenuated) have been developed by different agencies to prevent the spread of viral infection.

BHV-1 is a typical α -herpesvirus having a double-stranded DNA genome packaged in an icosahedral capsid composed of 150 hexameric, and 12 pentameric capsomers. The nucleocapsid is embedded in a proteinaceous layer known as the tegument, which is in turn surrounded by a lipid envelope, derived from host cell nuclear membrane.

The genome of BHV-1 contains 137kb of linear double stranded DNA, organised as, long (UL; 105kb) and short (US; 11kb) segments of unique DNA sequence (Mayfield *et al.*, 1983). US sequence is flanked by internal and terminal inverted repeat sequences of 12kb. The repeated elements enable US sequences to invert orientation with respect to the L segment, giving two isomeric form of the BHV-1 genome (Mayfield *et al.*, 1983).

As with other herpes viruses, the replication and assembly of BHV-1 occurs in the nucleus of the infected cell. The BHV-1 genome is sequentially transcribed in three waves in a cascading manner. These three class of genes are; immediate early (IE), early (E), and late (L). Expression of IE genes does not require the expression of other viral proteins. The expression of E genes, however, is dependent on the prior expression of IE proteins, while expression of the L genes is dependent upon viral DNA synthesis and expression of E

1.3. BOVINE HERPESVIRUS TYPE 1 (BHV-1)

Bovine herpesvirus type-1, also known as infectious bovine rhinotracheitis virus has been associated with infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, infectious balanoposthitis, and abortion. Close contact is required for transmission of BHV-1 infection, which can result in subclinical, mild, or severe disease. Because BHV-1 infection is of economic importance, several of vaccines (mostly live attenuated) have been developed by different agencies to prevent the spread of viral infection.

BHV-1 is a typical α -herpesvirus having a double-stranded DNA genome packaged in an icosahedral capsid composed of 120 hexamers, and 12 pentameric capsomers. The

gB; gC etc is a terminology relating to the homologous gene products in HSV-1.

surrounded by a lipid envelope, derived from host cell nuclear membrane.

The genome of BHV-1 contains 137kb of linear double stranded DNA, organised as long (UL; 102kb) and short (US; 11kb) segments of unique DNA sequence (Mayfield et al., 1983). US sequence is flanked by internal and terminal inverted repeat sequences of 12kb. The repeated elements enable US sequences to invert orientation with respect to the L segment, giving two isomeric forms of the BHV-1 genome (Mayfield et al., 1983).

As with other herpes viruses, the replication and assembly of BHV-1 occurs in the nucleus of the infected cell. The BHV-1 genome is sequentially transcribed in three waves in a cascading manner. These three classes of genes are: immediate early (IE), early (E), and late (L). Expression of IE genes does not require the expression of other viral proteins. The expression of E genes, however, is dependant on the prior expression of IE proteins, while expression of the L genes is dependant upon viral DNA synthesis and expression of E

genes (Misra *et al.*, 1981). Viral DNA synthesis is dependent upon the synthesis of early gene products, which include the proteins required for viral DNA synthesis (eg. viral DNA polymerase).

There may be as many as 74 open reading frames in the BHV-1 genome. Writh *et al.*, (1989) have identified the temporal distribution of 54 BHV-1 transcripts; 4 immediate early (IE), 21 early (E), and 12 late (L). The classification of the remaining 17 transcripts could not be determined (Writh *et al.*, 1989). Misra *et al.*, (1981) have identified up to 33 BHV-1 structural polypeptides, of which 11 were identified as glycoproteins. In addition to the structural proteins, 15 non-structural protein were also identified in BHV-1 infected cells (Misra *et al.*, 1981).

Four major glycoproteins, involved in the formation of the projecting spikes observed on the viral envelope have been identified; gI (gB), gII (gE), gIII (gC), and gIV (gD) (Collins *et al.*, 1984). gI consist of combination of three glycoproteins; 130K, 74K, and 55K, gII is a 108K glycoprotein, gIII is a 91K glycoprotein (also existing as a dimer with a molecular weight of 180K), while gIV is a 140K/71K dimeric protein (Van Drunen Little- Van den ^{Hurk} and Babiuk, 1986). These glycoproteins have an essential role in binding of the virus to the cellular receptor (attachment), and virus penetration [gI, gIII, and gIV are involved in virus attachment, with gIII playing a predominant role (Liang, *et al.*, 1991), while gIV is essential for virus penetration (Fehler, *et al.*, 1992)]. The four major glycoproteins are also important in induction of an immune response to BHV-1 infection (both humoral and cellular response). These glycoproteins have been used individually, or in combination, as subunit vaccine against BHV-1 (Babiuk *et al.*, 1987; Marshall *et al.*,

1986; Collins *et al.*, 1984). However, neither vaccines, nor natural infection protects against establishment of BHV-1 latent infection.

Another protein involved in induction of an immune response from BHV-1 infected cells is VP8. The VP8 is a 742 amino acid phosphoprotein, and is a major constituent protein in the tegument of virus particle. This protein is present in the nucleus of infected cells as early as 2h postinfection, suggesting that the protein might have some regulatory function in IE gene expression (Van Drunen Little-Van Den ^{HurK} _^, 1995). VP8 has homology to HSV-UL47, with protein kinase activity and is thought to be involved in the phosphorylation of BHV-1 tegument proteins (Carpenter and Misra 1991), however, its precise role is unclear.

1.4. PURPOSE AND AIM OF THE PROJECT.

The work described here further investigates the mechanism by which triterpenoid compounds exert an anti-retrovirus effect. The antiviral activity of triterpenoid compounds on the replication of two non-human retroviruses (FIV and FeLV-A) and a herpesvirus (BHV-1) was studied and compared. The aim was to identify cell lines resistant to the drug and to use these cell lines to investigate the effect of triterpenoid compounds on FIV and FeLV-A replication at the level of infectious virus yield and at the level of viral protein synthesis. The data generated should illuminate the mechanism of antiviral action.

2. CHAPTER TWO

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. CHEMICALS

The following chemicals were obtained from the Sigma Chemical Company (U.K.):

Piccoli type 400.

Bovine serum albumin conjugated to protein-A sepharose beads.

Haemocyanin from Keyhole limpet.

Hydrogen peroxide (30% w/w) solution.

Polybrene [Hexadimethrine bromide (1, 5-dimethyl-1, 5-diazasundecanemethylene

polymethobromide)].

Haemotoxiline was obtained from Difco, while Cyatogen bromide activated

sepharose 4B was obtained from Pharmacia.

2. CHAPTER TWO

All tissue culture **MATERIALS AND METHODS** methionine (E met) or

inorganic phosphate (EP) were supplied by Gibco-Life science technologies, Paisley

Scotland. E met and EP were produced by the Institute's Media department.

2.1.2. FINE CHEMICALS

Ciclooxolone sodium (CCK) and Carbamazepine were prepared by the Institute

by Miss L. Baxendale and Dr. E. Thornton of the Biotech Laboratory, Glaxo Wellcome

(London, U.K.).

Glycyrrhizic acid ammonium salt was purchased from Fluka.

The following chemicals were obtained from the Sigma Chemical Company:

Ouabain octahydrate [4β, 20 (21'-acetonide-1β, 20'-ene, 19α, 15'-epoxide-3β, 21'-

deoxy-α-L-mannopyranosyl); G-strophanthin].

2.1. MATERIALS

2.1.1. CHEMICALS.

The following chemicals were obtained from the Sigma Chemical Company (U.K):

Ficoll type 400.

Bovine serum albumin conjugated to protein-A sepharose beads.

Haemocyanin from Keyhole limpet.

Hydrogen peroxide (30% w/w) solution.

Polybrene [Hexadimethrine bromide (1, 5-dimethyl-1, 5-diazaundecamethylene polymethobromide)].

Haemotoxylene was obtained from Difco, while Cyanogen bromide-activated sepharose 4B was obtained from Pharmacia.

All tissue culture solutions except Eagle's medium lacking methionine (E met⁻) or inorganic phosphate (EP⁻) were supplied by Gibco-Life science technologies, Paisley, Scotland. E met⁻ and EP⁻ were produced by the Institute's Media department.

2.1.2. FINE CHEMICALS

Cicloxolone sodium (CCX) and Carbenoxolone sodium (CBX) were kindly supplied by Miss L. Baxendale and Dr. P. Thornton of the Biorex Laboratories limited company (London, U.K.).

Glycyrrhizic acid ammonium salt was purchased from Fluka.

The following chemicals were obtained from the Sigma Chemical Company:

Ouabain octahydrate [4 β , 20 (22)-cardenolide-1 β , 3 β , 5 α , 11 α , 14, 19-hexol-3 (6-deoxy- α 1-L-mannopyranosyl); G-stopphanthin].

H₇ [1-(5-Isoquinolinylsulfonyl)-2-methyl piperazine].

2.1.4. 3-amino-9-ethyl-carbazole.

Monensin (product, M5273).

Tunicamycin (product, T7765).

DAMP [3-(2, 4-dinitroanilino)-3'-amino-N-methyl-dipropylamine] (product, D09), was bought from Oxford Biomedical research, INC. (USA).

Horseradish peroxidase streptavidine (product, SA-5004) was purchased from Vector Laboratories (USA).

2.1.3. **ANTIBODIES**

2.1.5. The FIV antiserum from infected cats; and the monoclonal antibody (IE2) directed against FIV p24 (Pederson et al, 1990) were generously supplied by Dr. Robert Osborne, Veterinary School, University of Glasgow. Biotinylated horse anti-mouse IgG (H+L) (product, BA-2000) was obtained from Vector laboratories (USA).

Monoclonal antibodies directed against the p58 (product, G-2404) and β -COP (product, G-2279) Golgi marker proteins; the anti-mouse IgG (FC- specific) FITC conjugate (product, F-5387), and the anti-rabbit IgG (whole molecule) FITC conjugate (product, F-0511) were purchased from the Sigma Chemical Company.

Protein-A purified, anti-dinitrophenol (anti-DNP) monoclonal antibody (product, D09) was obtained from Oxford Biochemical Research, INC. (USA).

and used for radioactive labelling experiments.

RPMI 1640 medium lacking either D-Thucose RPMI glucose

Methionine (RPMI/ cysteine/methionine) supplemented with 25% fetal calf serum

2.1.4. RADIOCHEMICALS

All radiochemicals were purchased from Amersham International PLC, Amersham Bucks, England:

[³²P]-inorganic orthophosphate (specific activity 10 mci/ml) (product, PB S11).

L-[³⁵S] Methionine (specific activity, 1000 ci/mmol) (product, SJ 123).

[³⁵S] Cysteine (specific activity, 1000 ci/mmol) (product, SJ 232).

D-[1-¹⁴C] Glucosamine hydrochloride (specific activity, 54 mci/mmol) (product, CFA. 475).

2.1.5. MEDIA

Dulbecco's MEM containing 25mM hepes (DMEMh) supplemented with; 2% (4mM) L-glutamine, 2% penicillin-streptomycin (i.e. 20 units/ml penicillin, 20µg/ml streptomycin), 1% non-essential amino acids, 1% (1mM) sodium pyruvate, and 10% foetal calf serum (FC); (DMEMh 10% FC) was used for growth of FeA and QN10S cells.

Dulbecco's modified Eagle's medium (DMEgM) supplemented with, 2% (4mM) L-glutamine, 2% penicillin-streptomycin, 1% nonessential amino acids, and 10% FC (DMEgM 10% FC) was used for the growth of CrFK and MDBK cells.

Glasgow Eagle's medium (Eg) lacking either methionine or phosphate, as appropriate, was supplemented with 2% FC (Eg met 2% FC, or Eg p 2% FC respectively) and used for radioactive labelling experiments.

RPMI 1640 medium lacking either D-Glucose (RPMI glu⁻), or L-Cysteine and L-Methionine (RPMI cysteine/methionine⁻) supplemented with; 2% L-glutamine, 2%

penicillin-streptomycin, 1% nonessential amino acids, and 2% FC was used in the FIV and FeLV-A radioactive labelling experiments.

Bovine Embryo Kidney (BEK) cells and Bovine Embryo Lung (BEL) cells.

2.1.6. CELL CULTURES

Crandell Feline kidney (CrFK) cells: This epithelial cell line was the kind gift of Dr. Robert Osborne, Veterinary School, University of Glasgow, and was used for the propagation of the FIV virus. CrFK cell cultures were grown in DMEgM 10% FC and routinely passaged 1:6.

Feline Embryo Fibroblast (FeA) cells: This fibroblast cell line was used for the propagation of the FeLV-A virus, and were routinely passaged 1:4 in DMEMh 10% FC.

QN10S cells: This fibroblast cell line grown in DMEMh 10% FC medium, routinely subcultured 1:4, and was used only for the titration of FeLV-A. QN10S cells were derived from a clone of AH927 feline fibroblast cells into which the provirus of the Molony murine sarcoma virus had been introduced (O. Jarrett-unpublished data) and therefore harbour an endogenous virus. When QN10S cells are infected with FeLV-A, neither the FeLV-A nor the Molony murine sarcoma virus are capable of independent plaque formation, but the Molony murine sarcoma virus/FeLV-A pseudotype i.e. FeLV-A packaged in a Molony murine sarcoma virus envelope is able to plaque on the QN10S cell monolayers. QN10S and FeA cells were the gift of Professor O. Jarret Veterinary School, University of Glasgow.

Madin-Darby Bovine Kidney (MDBK) cells: This epithelial cell line was grown in DMEgM 10% FC medium and subcultured 1:6. The MDBK cell line was obtained from

Dr. D. Dargan and originally supplied by Dr. H.J. Field School of Veterinary Medicine University of Cambridge.

Bovine Embryo Kidney (BEK) cells and Bovine Embryo Lung (BEL) cells, were both grown in Eagle's 10% FC medium and passaged 1:4. These cell cultures were the kind gift of Dr. Richard Elliot, Institute of virology, University of Glasgow.

2.2.3. PREPARATION OF CICLOXOLONE, CARBENOXOLONE,

2.2 METHODS.

2.2.1. CELL CULTURE PASSAGE.

All cell lines were subcultured by washing the confluent cell monolayer twice with versene (0.6mm ethylenediaminetetra acetic acid , 1% Phenol Red) containing 10% trypsin, followed by incubation of the cells at 37°C for 5 minutes. The detached cells were resuspended in 20ml of the appropriate tissue culture medium, and re-seeded, at the appropriate cell density, in tissue culture flasks or dishes.

2.2.2. STORAGE AND RECOVERY OF FROZEN CELL CULTURES.

Cells were prepared for cryo-preservation by resuspending trypsinized cell cultures in storage medium (80% normal growth medium 10% DMSO, and 10% FC). The cell suspensions (2×10^6 cells/ml) was dispensed into 1ml aliquots in glass vials, and frozen slowly by incubating at -70°C overnight in a polystyrene container, after which the vials were transferred to the gaseous phase of a liquid nitrogen container (-170°C) for long term storage of the cells.

2.2.4. Cell suspensions were recovered from liquid nitrogen storage by thawing in a water bath at 37°C. The storage medium was then diluted 1:1 with tissue culture medium, added one drop, at a time, after which 2×10^6 cells were seeded on a small (75ml) tissue culture flask and incubated at 37°C in a CO₂ gassed incubator.

Cell viability was determined by trypan-blue dye exclusion:

2.2.3. PREPARATION OF CICLOXOLONE, CARBENOXOLONE, GLYCYRRHIZIN, MONENSIN, AND TUNICAMYCIN SOLUTIONS.

CCX (Mwt 668.8) and CBX (Mwt 614.7) were prepared as 10 mM solutions in sterile Mono-Q double-distilled ultra pure water, then diluted to 5mM with sterile 10% bovine serum albumin (BSA) fractionV (Sigma A-2153), BSA was sterilised by filtration through a 0.22μ Acrodisc (Gilman Science) filter. The BSA stabilises CCX and CBX in solution. CCX and CBX solutions were freshly prepared for each experiment.

GL was dissolved in a small volume of PBS (170mM NaCl, 3.4mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄) (pH was adjusted to 7.2 with 1.0M NaOH in order to allow the GL to dissolve), and then brought to a final concentration of 10mg/ml. The GL stock solution was sterilised by filtration through a 0.45μ and then a 0.22μ sterile Acrodisc filter, and stored at -20°C.

Monensin (MON) was prepared as a 10mM stock solution in ethanol and stored at -20°C.

Tunicamycin was prepared at 2mg/ml in DMSO and stored at -20°C.

2.2.4. THE EFFECT OF CCX, CBX OR GL ON FeA, ON10S, AND CrFK CELLS IN CULTURE.

2.2.4.1. DRUG CYTOTOXICITY TESTS.

Cell viability was determined by trypan-blue dye exclusion:

Trypan-blue dye solution was made by dissolving 0.5g of trypan-blue in 100ml of phosphate-buffered saline and filtering through a 0.45u Acrodisc filter (Gilman Science) before use.

FeA, CrFK, and QN10S cells were seeded at 5×10^5 cells/ 60mm tissue culture dish in DMEMh 10% FC, DMEgM 10% FC, or DMEMh 10% FC medium respectively. Following overnight incubation at 37°C, the culture medium overlays were replaced with the appropriate medium, either drug-free or containing various concentrations of CCX, CBX, or GL. Cell cultures were harvested for determination of cell viability and enumeration of the total cell number. At the time of drug addition (0h), and every day for a period of 10 days, the cultures were treated as follows:

1. The culture medium was decanted into a 15ml Falcon tube.
2. The monolayer was washed twice with trypsin/versene solution.
3. The washes were pooled with the growth medium in the Falcon tube.
4. The detached cells from the monolayer were pelleted by low speed centrifugation (~1000g./5 minutes/4°C).
5. The supernatant was discarded and the cell pellet resuspended in 1ml of tissue culture medium.

6. The resuspended cell pellet was pooled with, and used to resuspend, the cells from the trypsinised monolayer.
7. 100µl of the pooled cell suspension was mixed with 100µl of trypan blue dye, and the dye allowed to penetrate the cells for 5 minutes.
8. The total cell number and the number of blue-stained cells (non-viable) in each samples were counted microscopically using a Neubauer haemocytometer.

2.2.4.2. REVERSAL OF THE EFFECT OF CCX TREATMENT ON DRUG TREATED CELL CULTURES.

FeA and CrFK cell cultures were seeded at 5×10^5 cells/ 60mm tissue culture plates in DMEMh 10% FC or DMEgM 10% FC respectively. After overnight incubation at 37°C, the growth medium was replaced with the appropriate medium supplemented with 2% FC, and containing either no drug , or various concentrations of CCX. Sets of cell cultures were treated with the drug for 1, 2, or 3 days. One set of cultures were treated as follows: At the time of drug addition, and at daily intervals thereafter, the percentage cell viability and the total number of cells in each culture were measured. The medium was removed from the other sets of cultures at daily intervals, and the residual drug removed by washing with PBS 5% FC. These washed cell cultures were then overlaid with normal drug-free medium, and incubated at 37°C for a further five days, after which the total cell number and percentage cell viability were determined.

2.2.5. VIRUS CULTURES

2.2.5.1. VIRUSES

Feline immunodeficiency virus (FIV) was the gift of Dr. R. Osborne, and Professor O. Jarret.

Feline Leukaemia Virus type A (FeLV-A) was the gift of Professor O. Jarret, Veterinary School, University of Glasgow.

Bovine Herpes virus (BHV-1 strain 6666) was supplied by Dr D. Dargan and originally obtained from Dr. Hugh Field (School of Veterinary Medicine University of Cambridge).

2.2.5.2. PREPARATION OF VIRUS STOCKS.

2.2.5.2.1. BHV-1

Confluent monolayers of MDBK cells on 80oz roller bottles were infected with BHV-1 (strain 6666) at a m.o.i. of 0.01 p.f.u./cell. Following the virus absorption period (1h at 37°C) the inoculum was removed by washing with PBS, and the infected monolayer overlaid with DMEgM 10% FC. At 48h PI virus was harvested by scraping the cells into the medium with a rubber policeman, and sonicating the cell suspension. Cellular debris was pelleted, and the clarified virus stock stored at -70°C. BHV-1 stocks so prepared generally contained about 10^9 p.f.u./ml.

2.2.5.2.2. FeLV-A AND FIV.

FeA or CrFK cells were seeded at 2×10^7 cells/roller bottle and incubated overnight at 37°C . Sub-confluent monolayers of FeA or CrFK cells were infected with 1.42×10^7 p.f.u./roller bottle FeLV-A or 1×10^3 FIV p.f.u./roller bottle in 40ml of the appropriate medium respectively. Following 48-72h incubation at 37°C , each roller bottle was subcultured 1:6. The six infected roller bottle cultures were incubated at 37°C for 48h, after which the medium was decanted and replaced with 25ml of fresh medium. After a further 24h incubation at 37°C , the supernatant medium was decanted from the infected roller bottle cultures. The cellular debris was removed from the virus containing medium by centrifugation at 1000 g at 4°C for 10 minutes. The supernatant was collected, aliquoted, and stored at -70°C . FeLV-A stocks prepared in this way generally contained 5×10^6 to 1×10^7 p.f.u./ml, while FIV stocks yielded approximately 1×10^3 p.f.u./ml.

2.2.5.3. FeLV-A TITRATION.

QN10S cells were seeded at 2×10^5 cells/60mm tissue culture plate in 4 ml of DMEMh 10% FC and incubated overnight at 37°C . Serial ten-fold dilutions of FeLV-A were prepared in tissue culture medium containing 4ug /ml polybrene. The growth medium was decanted from the monolayers, which were then infected with 1ml of virus suspension and incubated at 37°C for 2 h, shaking the infected cell cultures every fifteen minutes to assist virus adsorption. The virus inoculum was washed from the cultures, and overlaid with tissue culture medium. The cultures were incubated at 37°C for 5 to 7 days in a well

humidified (90%) incubator, after which they were fixed , stained with Giemsa, and the virus plaques counted using a dissecting microscope (Figure I).

2.2.5.4. TITRATION AND IMMUNOSTAINING OF FIV PLAQUES ON CrFK CELL MONOLAYERS.

2.2.5.4.1. FIV TITRATION

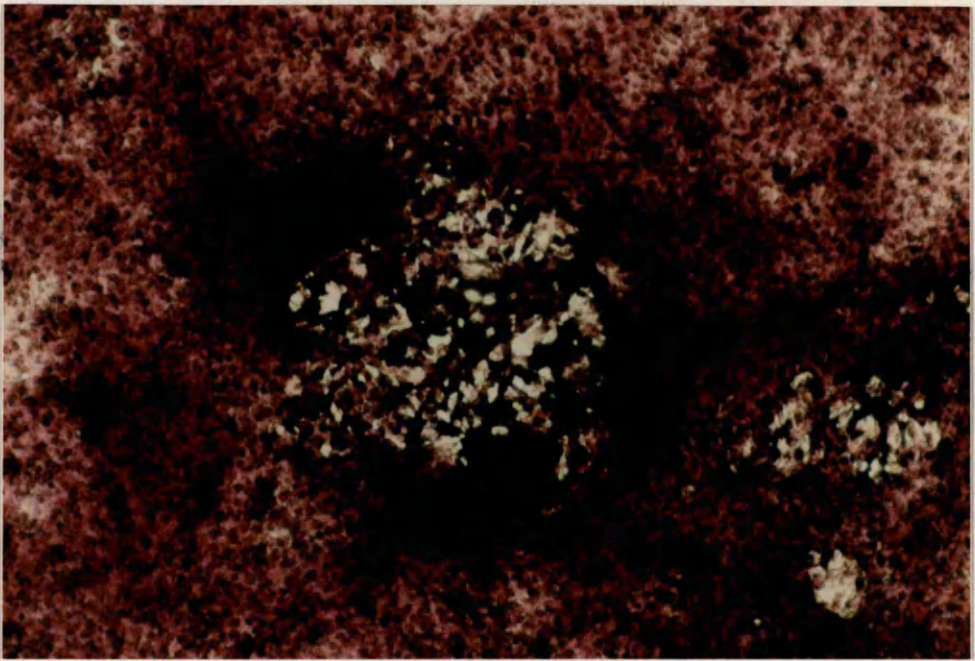
Crandell feline kidney cells (CrFK) were seeded at 1×10^5 cells/well on 24 well Nunc trays and incubated overnight at 37°C. Serial ten-fold dilutions of FIV stock were prepared in DMEgM 10% FC. The growth medium was decanted from the monolayers, which were then infected with 200µl of titrated FIV. After virus absorption (75 minutes at 37°C) the inoculum was removed, the cell monolayers washed 3 times with DMEgM 10% FC, and overlaid with one ml of DMEgM 10% FC.

The infected cell monolayers were incubated for a further 4 days at 37°C in a well humidified incubator (90%). The resulting virus plaques were identified by immunostaining using an FIV antiserum.

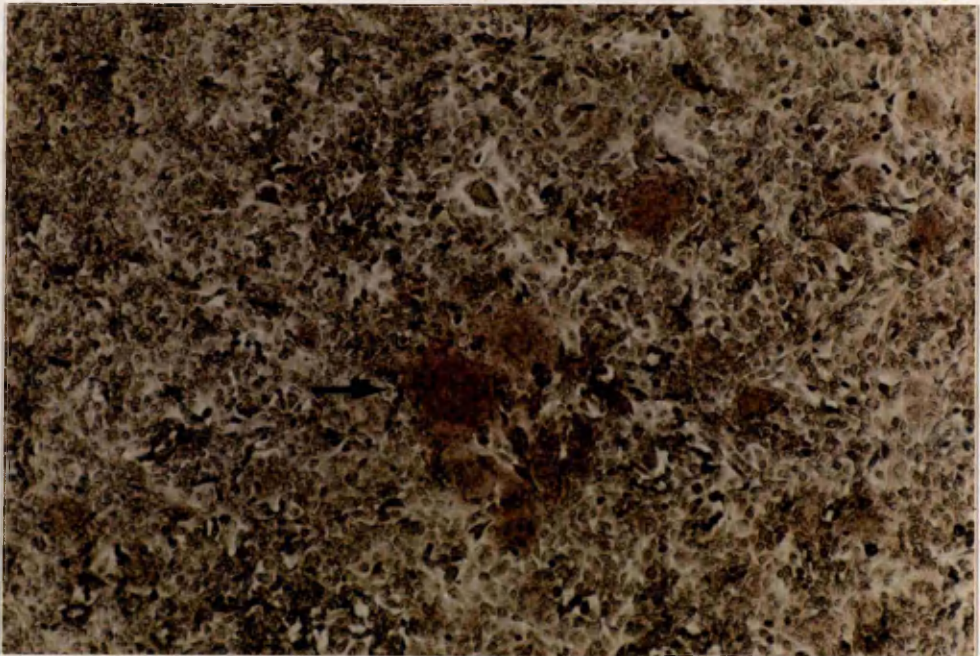
2.2.5.4.2. IMMUNOSTAINING

The medium from infected cell monolayers was emptied to a beaker containing chlorox, and the Nunc tray further evacuated by tapping against a surface covered with blotting paper. Cell monolayers were then fixed with 200µl of 1% acetic acid (Analar 99%) in ethanol for 20 minutes at RT. The monolayers were then washed 3 times with immunostaining buffer- tapping the Nunc tray to evacuate (as described), and then treated with 200µl of immunostaining buffer containing 5% heat-inactivated goat serum (blocking

FIGURE I.



FeLV-A plaque on FeA cells, fixed and stained with Giemsa.



**FIV plaque on CrFK cells immunostained and visualised with Streptavidine peroxidase and Aminoethyl-Carbazol.
Arrow shows typical FIV syncytium**

buffer) at 37°C for 60 minutes, or overnight at 4°C. After three cycles of washing with immunostaining buffer the infected cells were probed with a 1/4500 dilution of anti-FIV p24 monoclonal antibody (IE2)- shaking at 37°C for 75 minutes. The cell monolayers were then washed 3 times with immunostaining buffer and treated with 200µl of biotinylated horse anti-mouse serum (Vector) (1/3500 dilution in immunostaining buffer) for 15 minutes at 37°C. Following another three cycles of washing with immunostaining buffer the infected cell monolayers were treated with streptavidin/ peroxidase (Vector) (1/2250 dilution in immunostaining buffer) and incubated at 37°C for 15 minutes. Finally the cell monolayers were washed one last time with immunostaining buffer and then once with freshly prepared 0.05M acetate buffer pH. 4.8-5.0. The FIV plaques were visualised by staining with 200µl of amino-ethyl carbazole (AEC) staining solution. After 10 to 15 minutes incubation at RT a distinct red-brown coloration appeared over the FIV plaques. When the infected cell background began to show coloration, the reaction was stopped by immersing the cell layers in water. The cell monolayer was counter-stained with 200µl/well of Meyer's haematoxylin (diluted 1:1 with water) for 10 minutes at RT. Plates were allowed to air-dry after which the FIV plaques were counted using a dissecting microscope (Figure I).

2.2.5.5. BHV-1 TITRATION.

30mm tissue culture plates were seeded with MDBK cells at 1×10^6 cells/plate, and allowed to form a monolayer overnight. Serial ten-fold dilutions of BHV-1 stocks were

prepared in PBS+ 5% FC, and the cell monolayers infected with 100µl of virus dilution and incubated for 1h at 37°C. After virus absorption, the monolayers were washed 3 times and overlaid with DMEgM 10% FC. At 48h post-infection the cell monolayers were fixed and stained (24h at RT) with Giemsa (improved R66 stain solution). The stained cell layers were washed, dried, and the BHV-1 plaques counted using a dissecting microscope.

2.2.5.6. VIRUS PURIFICATION.

2.2.5.6.1. Virus concentration.

Cell cultures were infected with FeLV-A or FIV and crude virus stocks prepared as previously described (section 2.2.5.2.2). After clarification of the virus stock, the virus particles were pelleted by centrifugation at 12000 r. p. m. at 4°C for 3h in a Sorvall GSA rotor.

2.2.5.6.2. Gradient purification.

The pelleted FIV or FeLV-A virus particles were gently resuspended overnight in Eagles medium lacking phenol red (Epr⁻) (allowing 500µl Epr⁻/roller bottle). The virus particle suspension was layered onto Ficoll 400 gradients (5-15% made in Epr⁻) prepared in 37 ml Beckman ultra clear AH629 tubes. The virus particles were banded by centrifugation at 12000 r.p.m. for 3h at 4°C in a Sorvall AH629 rotor. The virus band was collected by side puncture of the tube. The purified virus fraction was then made to 37 ml with Epr⁻ and pelleted by centrifugation at 12000 r.p.m. in the Sorvall AH 629 rotor at 4°C. The pellet of purified virus was gently resuspended in Epr⁻ (50µl/roller bottle) and stored at -70°C.

2.2.6. SINGLE-CYCLE REPLICATION OF FeLV-A IN RESTING OR NON-RESTING FeA CELL CULTURES.

2.2.6.1. Non- Resting Cell cultures.

FeA cells were seeded in 24 well Nunc trays at 2×10^5 cells/well, and allowed to form a monolayer (16h at 37°C). The medium was then decanted and the cells infected with $100\mu\text{l}$ /well FeLV-A at a m.o.i. of 5 p.f.u./cell in the presence of $4\mu\text{g}/\text{ml}$ of polybrene. After virus absorption (2h at 37°C) the infected monolayers were washed three times with PBS, overlaid with DMEMh 10% FC, and incubated at 37°C . At 0, 3, 6, 9, 12, 18, 24, 36, and 48 h post-infection (PI), the virus yield was harvested by taking the supernatant of the infected monolayers, and clarifying it by low speed centrifugation ($1000\text{g}/10$ minutes/ 4°C). The virus yield was titrated on QN10S cell monolayers.

2.2.6.2. RESTING CELL CULTURES

60mm tissue culture plates were seeded with 5×10^5 FeA cells/plate in DMEMh 0.5% FC, incubated at 37°C and allowed to grow for seven days after which time the cell layer had appeared to have stopped growing. The conditioned medium from resting cell layers- depleted in growth factors- was decanted to a sterile flask and retained for later use. Resting cell monolayers were infected with FeLV-A at a m.o.i. of 5 p.f.u./cell in the presence of $4\mu\text{g}/\text{ml}$ of polybrene, using conditioned medium from extra resting cell cultures as the virus diluent. After the 2h virus adsorption period, the cells were washed three times

with PBS, overlaid with conditioned medium and incubated at 37°C. Infected cell culture medium was harvested at 0, 3, 6, 9, 12, 18, 24, 36, and 48 h PI, clarified by centrifugation (1000g for 10 minutes), and the infectious virus yield titrated on QN10S cell monolayers.

2.2.7. FELINE IMMUNODEFICIENCY VIRUS PLAQUE REDUCTION ASSAY.

CrFK cell monolayers were seeded on 24 well Nunc trays at 1×10^5 cells/well in one ml of DMEgM 10% FC. Following overnight incubation the growth medium was decanted and the cell layers infected with FIV at 100 p.f.u./well. The cells were allowed 75 minutes at 37°C to absorb the virus after which the inoculum was removed by washing three times with DMEgM 10% FC. The infected cell layers were then overlaid with DMEgM 10% FC containing various concentrations of either CCX, or CBX, and incubated at 37°C for 4 days, after which the cultures were fixed, and FIV plaques identified by immunostaining, as described previously.

2.2.8. DRUG INFECTIOUS VIRUS YIELD DOSE-RESPONSE EXPERIMENT.

2.2.8.1. FELV-A

FeA cells were seeded on 24 well Nunc tray at 2×10^5 cells/well and incubated overnight at 37°C. The growth medium was then decanted, and the monolayers infected with FeLV-A at a m.o.i. of 5 p.f.u./cell, and allowed 2h (shaking every 15 minutes) at 37°C to absorb the virus. The infected cell layers were washed three times with DMEMh 2% FC to remove unbound virus, overlaid with 1ml DMEMh 2% FC (containing various concentrations of CCX, CBX, or GL) and then incubated at 37°C for 48h. The cell-

released virus fraction (CR) was obtained from the supernatant of the tissue culture medium, clarified by low speed centrifugation (1000 g./10 minutes/4°C). The tissue culture medium pellet (containing cell debris and detached cells) was resuspended in 1ml of tissue culture medium and pooled with the cells remaining attached to the plate. The cell-associated (CA) virus fraction was obtained after scraping the infected cells into the 1ml resuspended cell pellet, and sonicating the cell suspension to release the cell-associated virus. The infectivity in CA and CR virus fractions were then titrated on QN10S cell monolayers.

2.2.8.2. **BHV-1.**

30 mm tissue culture plates were seeded with MDBK cells at 1×10^6 cells/plate and incubated overnight at 37°C. The resulting monolayers were infected with BHV-1 at a m.o.i. of 5 p.f.u./cell and allowed to absorb the virus for 1h at 37°C. The infected cell monolayers were then washed three times with DMEM 2% FC to remove unbound BHV-1, and overlaid with 2 ml of DMEM 2% FC, containing various concentrations of CCX, or CBX. The cells were then incubated at 37°C for 24h, after which the CA and CR virus yields were harvested (as previously described for FeLV-A). The infectious BHV-1 yield in both CA and CR virus fractions were titrated on MDBK cells.

(SDS PAGE)

Virus and cellular polyacrylamide were separated on polyacrylamide gels.

0.1% SDS. (Marsden, et al, 1976)

The ratio of acrylamide to bisacrylamide for all of the gels was 19:1.

The running gel contained 7.5%, 9%, or 10% acrylamide while the stacking gel contained

2.2.9. INVESTIGATION OF THE ANTIVIRAL MECHANISM OF CCX, CBX,

AND GL.

2.2.9.1. VIRUCIDAL ACTIVITY.

0.5 ml of FIV (1×10^3 p.f.u./ml), FeLV-A (1.4×10^7 p.f.u./ml), or BHV-1 (2.31×10^9 p.f.u./ml) were mixed with 0.5 ml tissue culture medium containing either no drug or increasing concentrations of CCX, CBX, or GL. 100ul of the drug- free virus suspension was withdrawn and titrated immediately (0h sample). The drug-free and drug-treated virus suspensions were each then split, and half the volume incubated at 4°C, and half at 37°C. The FIV, FeLV-A, and BHV-1 infectivity remaining in drug-free or drug-treated cultures after 24h incubation was then determined by titration. Although the numbers of infectious virus particles (FIV, FeLV-A, and BHV-1) varied in each case, the total protein content of the samples would have been much the same as the virus stocks were prepared in medium containing 10% foetal calf serum. Variation in the protein content of samples is therefore unlikely to have influenced the virucidal activity obtained for triterpenoid drugs against the different viruses.

2.2.9.2. POLYACRYLAMIDE GEL ELECTROPHORESIS OF POLYPEPTIDES

(SDS PAGE).

Virus and cellular polypeptides were separated on polyacrylamide gels in the presence of 0.1% SDS. (Marsden, et al, 1976)

The ratio of acrylamide to bisacrylamide for all of the gels was 37:5:1. The separating gel contained 7.5%, 9%, or 10% acrylamide, while the stacking gel contained

5% acrylamide. 4ml (10%) polyacrylamide minigels were used to resolve FIV and FeLV-A polypeptides, while 65ml (9%, or 10%) polyacrylamide gels were used for BHV-1 studies. 7.5% polyacrylamide gels were also used on occasion to resolve proteins of certain molecular weights.

SDS PAGE gel preparation and pouring were as described by Marsden et al (1976). After pouring, the resolving gel was overlaid with Butan-2-ol in order to obtain a smooth gel surface upon polymerisation (large gels 1-2h, minigels 15 minutes). The butan-2-ol was removed by washing with tap water, and the surface of the gel dried using a Whatman Seed test paper grade 182, before the stacking gel was poured and bonded to the resolving gel. Large gels were electrophoresed with a constant current of 60mA, while minigels were electrophoresed at 150volts at maximum current. The molecular weights of the viral or cellular polypeptides were estimated by comparison with Rainbow protein molecular weight markers (Amersham Life Science).

2.2.9.3. FIXING, STAINING, AND DESTAINING OF SDS PAGE GELS.

Polyacrylamide gels were fixed/stained with SDS PAGE fix/stain solution (see solutions) for a minimal period of 1h at RT. The polypeptide bands were then visualised by treating the gel with destain solution overnight, while gently agitating the gel on a shaker at RT.

Gels containing protein samples which were labelled, to a low level with radioactivity, were soaked in enhancer En³ Hance (New England Nuclead) for 1h at RT, washed three times in water, (10 minutes each), and then soaked in water for 30 minutes at

RT. After which the gel was vacuum dried (30 in/Hg, at 80°C for 2h) onto Whatman Seed test paper grade 182 using a Bio-Rad model 583 gel dryer.

Dried gels were exposed to X-OMAT S film at RT for varying periods of time then developed in a Kodak X-OMAT Processor model ME-3. The gel was then fixed by treatment (for 1h at RT with (30% ethanol, 0.5M NaOAc, 0.5% glutaraldehyde, and 0.2% sodium thiosulfate). The gel was then washed 3 times with double-distilled water, and impregnated with 0.1% silver nitrate, in 0.02% formaldehyde for 30 minutes at RT. Protein bands in the gel were visualised after colour development following (5-15 minutes) treatment with a solution of 25% Na_2CO_3 and 0.01 % formaldehyde. Colour development was stopped by soaking of the gel in a solution of 0.05% EDTA for 5 minutes. To obtain a permanent copy of the silver-stained gel, the gel was washed in distilled water and photographically reproduced by contact printing using X-OMAT duplication film and a Wardley duplicator machine.

2.2.9.5. WESTERN IMMUNOBLOTTING

Protein extracts were separated on 10% polyacrylamide gels using the General minigel electrophoresis apparatus. The banded proteins were then transferred to ECL nitrocellulose membrane (Amersham) by electroblotting. The membrane was then incubated overnight at 4°C on a rotary shaker with blocking buffer (0.05% Tween 20 in PBS) to reduce non-specific binding of the primary antibody. The blocking buffer was removed by three washes in PBS +0.05% Tween 20 for 5 minutes. The membrane then probed with antibody (either anti-HIV or anti-HelV) for 1 hour at 4°C. After three washes in PBS +0.05% Tween 20; 1% BSA, in PBS) overnight at 4°C. After the secondary antibody was decanted, the membrane was washed three times in 0.05% Tween 20 in PBS for 5 minutes.

2.2.9.4. SILVER-STAINING OF PROTEINS IN SDS PAGE GELS.

Polyacrylamide gels containing non-radioactive protein samples from uninfected or virus infected cells were treated with ethanol, acetic acid, and water mixture (at a ratio of 30:10:60) for by shaking gently at RT for 1h. The gel was then fixed by treatment for 1h at RT with (30% ethanol, 0.5M NaOAC, 0.5% gluteraldehyde, and 0.2% sodium thiosulfate). The gel was then washed 3 times with double-distilled water, and impregnated with 0.1% silver nitrate, in 0.02% formaldehyde for 30 minutes at RT. Protein bands in the gel were visualised after colour development following (5-15 minutes) treatment with a solution of 25% Na_2CO_3 , and 0.01 % formaldehyde. Colour development was stopped by soaking of the gel in a solution of 0.05% EDTA for 5 minutes. To obtain a permanent copy of the silver-stained gel, the gel was washed in distilled water and photographically reproduced by contact printing using X-OMAT duplication film and a Wardray duplicator machine.

2.2.9.6. IMMUNOPRECIPITATION.

2.2.9.5. WESTERN IMMUNOBLOTTING.

Protein extracts were separated on 10% polyacrylamide gels, using the Biorad minigel electrophoresis apparatus. The banded proteins were then transferred to Hybound-ECL nitrocellulose membrane (Amersham) by electro-blotting (50v for 1h). The membrane was then incubated overnight at 4°C on a rotary shaker with blocking buffer (5% Marvel™, 0.05% Tween 20 in PBS) to reduce non-specific binding of the antibodies. Excess blocking buffer was removed by three washes in PBS +0.05% Tween 20 (15 minutes each), and the membrane then probed with antibody (either anti-FIV or anti FeLV-A 1/50 dilution in 0.05% Tween 20; 1% BSA, in PBS) overnight at 4°C. After the antibody solution was decanted, the membrane was washed three times in 0.05% Tween 20 in PBS (15 minutes

each), incubated for 1h at 37°C with protein-A/ horse- raddish peroxidase (diluted 1/1000 in PBS containing 0.05% Tween 20, and 1% BSA) and then washed twice (15 minutes each) with 0.05% Tween 20 in PBS. Protein bands tagged with virus specific antibody were visualised using enhanced chemoluminescence (ECL) western-blot detection solution (Amersham Life Science) as follows:

1. Equal volumes of ECL solutions 1 and 2 were mixed together and pipetted gently over the blots for approximately 1 minute.
2. The membrane was removed from the ECL solution, placed between two sheets of Melanex film, and exposed to X-OMAT S film for a range of time to obtain short, medium, and long exposures of the blot..
3. The film was then developed using Kodak X-OMAT Processor model ME-3.

2.2.9.6. IMMUNOPRECIPITATION.

2.2.9.6.1. PREPARATION OF PROTEIN EXTRACTS FOR IMMUNOPRECIPITATION.

The growth medium was removed from virus infected cells, they were then washed twice with PBS, and a protein extract prepared by addition of the extraction buffer. The growth medium from the infected cell layer was clarified by low speed centrifugation, and the virus particles pelleted from the clarified supernatant by centrifugation at 20,000 r.p.m. for 2h (4°) in a Sorvall SS-34 rotor. A protein extract of the pelleted particles was then prepared by the addition of the extraction buffer.

2.2.9.6.2. PREPARATION OF PROTEIN-A SEPHAROSE BEADS

1. 0.2 g of protein-A sepharose beads were placed in a 15ml Falcon tube, and soaked in 10-15ml of the immunoprecipitation extraction buffer for 15 minutes.
2. The swollen beads were washed three times in the extraction buffer, pelleting the beads after each wash by centrifugation at 1000 r.p.m. for 5 minutes at 4°C.
3. After the final wash, the pelleted beads were resuspended in a volume of the extraction buffer equal to the volume of the pellet. The resuspended stock of protein A sepharose beads was kept at 4°C.

2.2.9.6.3. IMMUNOPRECIPITATION REACTION.

1. 50 µl of infected cell extract, or the pelleted virus extract, were mixed with FIV or FeLV-A infected cat sera, as appropriate (each at 1/20 dilution) in an Eppendorff tube, and incubated at 4°C overnight on a rotary mixer.
2. 60ul of protein-A sepharose beads were added to each antibody/antigen mixture, and incubated for 1h at 4°C on a rotary mixer at RT.
3. 600ul of wash buffer (0.6M lithium chloride, 0.1M Tris pH 8, 1.4M mercaptoethanol) was added to each sample .
4. Samples were microfuged at 3000 r.p.m. for 30 sec at room temperature.
5. Supernatant was removed, and 600ul of wash buffer was added to each pellet.
6. The pellet was gently resuspended by inversion of the tube.
7. The cycle was repeated four times

2.2.9 After the final wash the supernatant was removed from the pelleted beads, and 20ul of SDS PAGE extraction buffer was added to each pellet. The immunoprecipitated proteins were eluted from the beads by boiling for five minutes, after which the beads were pelleted and the supernatant containing the eluted proteins examined by SDS PAGE.

2.2.9.7. RADIOLABELING OF VIRUS AND CELLULAR PROTEINS.

2.2.9.7.1. [¹⁴C]-GLUCOSAMINE LABELLING OF MOCK-INFECTED AND FIV

SPECIFIC POLYPEPTIDES AND PELLETING OF THE VIRUS

PARTICLES MADE IN THE PRESENCE OF CCX.

60mm tissue culture plates were seeded with third passage persistently infected cells at 5×10^5 cells/plate. After overnight incubation the growth medium was replaced with glucose-free RPMI 1640 + 2% FC (either drug-free or containing CCX). Following further incubation for 30 minutes at 37°C, the persistently infected cell cultures were washed three times with glucose-free medium, and finally overlaid with RPMI 1640 (either drug-free or containing CCX). The cultures were then labelled 3h to 48h after drug addition, with ¹⁴C glucosamine (5μci/μl) (Hosie and Jarrett, 1990) after which the tissue culture medium was decanted and centrifuged to pellet the cell-free virus. The cell monolayers and the pelleted virus particles were lysed with the extraction buffer, and used in immunoprecipitation reactions with anti-FIV serum.

2.2.9.7.2. [³⁵S]-METHIONINE/CYSTEINE LABELLING OF MOCK-INFECTED AND FELV-A OR FIV INFECTED CELL POLYPEPTIDES, AND PELLETING OF THE VIRUS PARTICLES MADE IN THE PRESENCE OF CCX.

Third passage, FIV persistently infected CrFK cell monolayers or third passage FeLV-A persistently infected FeA cells were seeded at either 5×10^5 cells/60mm plate or 2×10^5 cells/well in 24 well Nunc trays. Following overnight incubation at 37°C, the tissue culture medium was replaced by RPMI 1640 2% FC lacking methionine and cysteine (either drug-free or containing CCX). Cell cultures were pre-treated with CCX for 30 minutes at 37°C. Drug-treated cultures were then washed 3 times, and overlaid with methionine/cysteine-free medium (either drug-free or containing CCX). ³⁵S meth/cys mixture (50μci/ml) was added to the cell culture at 3h after drug addition, after which the monolayers were incubated at 37°C until 48h after drug addition. The infected cell monolayers were then harvested as follows:

1. The culture medium was removed and clarified by centrifugation at 2000 r.p.m/ 30 minutes.
2. The supernatant was filtered through a 0.22μ Acrodisc filter, and centrifuged at 12000 r.p.m. for 3h in the TST 41 rotor to pellet the virus particles.
3. The virus particle protein extract was prepared by resuspension of virus particles in 50ul of SDS PAGE extraction buffer.
4. The infected cell extract was prepared by adding 250ul of SDS PAGE extraction buffer to the infected cell layers.

5. Protein extracts were boiled for 3 minutes, after which 10ul samples were spotted onto Whatman #1 filter discs. The discs were washed twice in ice-cold 5% TCA, twice in 100% ethanol at RT, and allowed to air dry at RT. The filters were then placed in plastic vials containing 5 ml of Ecoscint (National Diagnostics) and the amount of the radioactivity in each sample determined by liquid scintillation counting using a Beckman model LS 5000 CE Scintillation Counter.
6. The radioactivity contained in each samples was standardised. Protein extracts containing equal CPM were loaded into each gel slot, and electrophoresed on SDS PAGE gels at 150 volts.
7. Finally the gel was fixed/stained, dried, and autoradiographed.

2.2.10. ELECTRON MICROSCOPY.

2.2.9.7.3. [³⁵S]-METHIONINE AND [³²P]-INORGANIC PHOSPHATE LABELLING OF MOCK-INFECTED AND BHV-1 INFECTED CELL POLYPEPTIDES.

2.2.10.1. NEGATIVE STAINING.

Monolayers of MDBK cells (3×10^5 cells/well on 24 well Nunc trays) were either mock-infected (MI) or infected with BHV-1 at a m.o.i. of 20 p.f.u./cell. After virus absorption (1h at 37°C), the cell monolayers were washed three times, and overlaid with either methionine-free or phosphate-free Eagles medium 2% FC as appropriate (either drug-free or containing various concentrations of CCX). [³⁵S]-methionine (10 µci/ml) or [³²P]-orthophosphate (50µci/ml) was added to each cell culture at 3h or 4h PI respectively. Following overnight labelling, the infected cell monolayers were harvested as follows:

The [³⁵S] methionine-containing tissue culture overlay was discarded and the cells washed once with PBS. The infected cell protein extract was prepared by the addition of 250µl of SDS PAGE extraction buffer to the monolayers.

The [³²P] inorganic phosphate containing overlay was discarded and each cell layer quickly and carefully washed with distilled water, then lysed with 200µl of distilled water. 250µl of DNAase/RNAase mixture (50µg/ml DNAase; 50µg RNAase) was then added to each lysed sample which was then incubated at 37°C for 15 minutes. 150µl of SDS PAGE extraction buffer was then added to each lysed cell preparation. Protein extracts were electrophoresed on 9% SDS-PAGE gels. The gel was finally fixed/ stained, dried, and autoradiographed.

2.2.10. ELECTRON MICROSCOPY.

2.2.10.1. NEGATIVE STAINING.

5µl of virus suspension was mixed with 5µl of sodium silico tungstate, containing latex beads at 1.43×10^{10} /ml. The virus/bead suspension was incubated for 2 minutes at RT, then 5µl was applied to a cellulose nitrate coated EM grid and allowed to stand for 2 minutes. Excess sample was removed by blotting with a piece of Whatman # 1 filter paper. The virus/latex bead coated grid was then examined with a GL 100S electron microscope operating at 60KV and at the magnification of 40,000 times.

2.2.10.2. EPOXY-RESIN (EPON 812) EMBEDDING AND THIN SECTIONING OF TISSUE CULTURE SAMPLES

30mm tissue culture plates were seeded with MDBK cells at 1×10^6 cells/plate, and incubated overnight at 37°C. The cell layers were infected with BHV-1 at a m.o.i. of 5 p.f.u./cell, and allowed 1h at 37°C to absorb the virus. Infected cultures were washed 3 times in DMEgM 2% FC and overlaid with DMEgM 2% FC containing various concentrations of CCX. The growth medium was removed after 24h incubation at 37°C, and the cell layers harvested by scraping into 0.5ml of PBS. The cell suspension was pelleted at 9000 r.p.m. for 5 minutes in the MSE microfuge. The cell pellet was fixed with 0.5 ml of 2.5% gluteraldehyde (EM grade) in PBS, and stored overnight at 4°C. After three washes with PBS, the pellet was post-fixed and stained with 1% osmium tetroxide in PBS for 2h. Following further washes in PBS the cell pellet was dehydrated by sequential treatment with increasing concentrations of ethanol for ranging period of time (30% 3h, 50% 3h, 70% 18h, 90% 3h, 100% 18h). The cells were then permeated with Epon:ethanol (1:1), at RT overnight, after which, the cells were embedded in fresh, undiluted, Epon which was polymerised by baking at 60°C for 48h.

Thin sections approximately 100nm thick were, cut from the cell pellet using a Richert Jung ultramicrotome fitted with a diamond knife. Thin sections were stained with uranyl acetate in 50% ethanol for at least 1h, after which the sections were washed in double-distilled water. The thin sections were counter-stained with lead citrate for 5 minutes, washed in double-distilled water, and examined in a GL 100S electron microscope operating at 60KV and at a magnification of 20,000 to 40,000 times.

2.2.11. PRECLEANING OF ANTI-MOUSE-FITC-CONJUGATE.

The anti-mouse FITC conjugate purchased from the Sigma Chemical Company consistently gave non-specific labelling of cells in the absence of primary antibody. To remove this non-specific activity, 30mm tissue culture plates were seeded, overnight with MDBK cells at concentration of 2×10^6 cells/plate. The resulting monolayers were fixed in 90% ethanol in PBS for 4 minutes at RT, washed 3 times (5 minutes each) in PBS, overlaid with 1 ml of anti-mouse FITC conjugate (diluted 1/20 in PBS), and incubated for 1h on a shaker at RT. The anti-mouse FITC conjugate was then pipetted off, and centrifuged at 1000-1500g for 10 minutes at 4°C to pellet cellular debris. The anti-mouse FITC-conjugate was then tested on fresh monolayers of uninfected MDBK cells, to check for non-specific labelling. If fluorescence was detected the cycle of cleaning was repeated (three pre-cleaning steps were generally sufficient).

2.2.12. DETECTION OF ACIDIC COMPARTMENTS IN CCX-TREATED CELLS BY IMMUNOFLUORESCENCE USING THE DAMP/ANTI-DAMP

2.2.12. DETECTION OF GOLGI APPARATUS IN CCX TREATED CELLS BY IMMUNOFLUORESCENCE USING ANTI-GOLGI ANTIBODIES AS MARKERS.

13mm glass coverslips were de-greased by boiling in undiluted chloros for 3 minutes, washing in DI water, rinsing with 100% ethanol followed by air drying. Each coverslip was then polished using a Whatman lens tissue. Finally the coverslips were sterilised by dry-oven baking at 160°C for 2h.

MDBK or CrFK cells were seeded at 2×10^5 cells/well on glass cover slips in 24 well Nunc trays. After overnight incubation at 37°C, the growth medium was replaced with medium containing DAMP, or medium containing CCX, and the cells incubated for 5h at 37°C.

The cell layers were then washed three times (5 minutes, each) with PBS and fixed/permeabilized, at RT, with 90% ethanol in PBS (4 minutes). The fixed cell layers were washed 3 times (10 minutes, each on a shaker) with PBS, and then treated with the primary antibody [anti-P58 (1/50), or β COP (1/20) in PBS] for 60 minutes at RT. The cells were again washed 3 times with PBS, then treated for 60 minutes with the secondary antibody (pre-cleaned anti-mouse IgG-FITC conjugate diluted 1/80). Finally the monolayers were again washed 3 times with PBS, and then mounted on glass slides using (CITIFLUOR mounting solution, and nail varnish was used to seal the edges of the coverslip). The cells were then examined for fluorescence under UV illumination using a Nikon microphot-SA fluorescent microscope.

2.2.14. INVESTIGATION OF THE EFFECT OF OVARAIN IN COMBINATION

2.2.13. DETECTION OF ACIDIC COMPARTMENTS IN CCX-TREATED CELLS BY IMMUNOFLUORESCENCE USING THE DAMP ANTI-DAMP SYSTEM.

MDBK cells were seeded at 2×10^5 cells/well on sterile 13mm glass coverslips in a 24 well Nunc tray and allowed overnight to form a monolayer. The medium decanted and replaced either with fresh drug-free medium or medium containing CCX , and incubation at 37°C continued for 5h. Cell cultures were then washed 3 times with PBS (10 minutes, each), before adding growth medium containing 30uM [3-(2,4-dinitroanilino)-3'- aminoN-methyl-dipropylamine] (DAMP) and continuing incubation at 37°C for 30 minutes. The medium containing DAMP was then decanted, and the cell cultures fixed with 3% paraformaldehyde for 15 minutes at RT. The cell layers were then washed once (10

minutes) in 50 mM ammonium chloride and twice (10 minutes, each) in PBS. The cell layers were pre-cooled and kept on ice prior to, and during, permeabilization with 0.1% triton X-100 for 5 minutes. The permeabilized cells were then incubated with 3% BSA in PBS at, RT, for 15 minutes, and then treated with the primary antibody (1/10 dilution of anti-DNP antibody in 0.2% BSA/PBS) for 1h at 37°C. The monolayers were washed 3 times with 0.2% BSA/PBS (10 minutes, each), and then treated with the secondary antibody (pre-cleaned anti-mouse IgG-FITC conjugate diluted 1/80) for 1h at 37°C. The monolayers were finally washed 3 times again with PBS, and mounted on glass slides (using CITIFLUOR mounting solution). The cell layers were then examined for fluorescence under UV illumination using a Nikon microphot-SA fluorescent microscope.

2.2.14. INVESTIGATION OF THE EFFECT OF OUABAIN IN COMBINATION WITH CCX ON P58 GOLGI STAINING OF MDBK CELLS.

MDBK cells were seeded at 2×10^5 cells/well on coverslips in 24 well Nunc trays. After overnight incubation at 37°C, the growth medium was replaced with either drug-free medium, medium containing 300µM CCX (4h), or medium containing 25µM Ouabain (1h). Additional cell layers which had been treated with 25µM Ouabain for 1h were, then treated with 300µM CCX for 4h at 37°C. The cells were washed three times with PBS (5 minutes each) and fixed/permeabilized at RT with 90% ethanol in PBS for 4 minutes. Fixed cell layers were washed with PBS three times (10 minutes each on a shaker) then treated with the primary antibody (anti-P58 diluted 1/50) for 60 minutes at RT. The cells were again washed three times with PBS, and then treated for 60 minutes with the secondary antibody (pre-cleaned anti-mouse IgG-FITC conjugate diluted 1/80), after which

the cells were again washed and the coverslips mounted on a glass slide. The cells were studied for fluorescence under UV illumination using a Nikon microphot-SA fluorescent microscope.

2.2.15. PREPARATION OF A RABBIT ANTIBODY DIRECTED AGAINST CCX.

2.2.15.1. PREPARATION OF CCX-BSA AND CCX-KLH CONJUGATE.

In order to increase the probability of obtaining an antibody against CCX (Mwt 668.8) the drug was conjugated to BSA, or to Keyhole Limpet Haemocyanin (KLH) (ELISA). EDC was used to form a covalent bond between an amino group on BSA or KLH, and the COONa group on CCX. 20 μ Mole of CCX was mixed with 600 μ l of 0.33M EDC by stirring in the dark for 5 minutes. 400 μ l of BSA solution (20mg/ml) was then added and allowed to mix overnight at RT.

In order to monitor the coupling of CCX to BSA a small amount of ^3H CCX was included in the reaction. After overnight mixing the CCX/BSA/EDC mixture was dialysed against; double distilled water for 5-6h at RT, followed by dialysis against 1% acetic acid overnight at RT, and finally dialysed against 3 changes of PBS over 2 days at 4°C. The dialysed material was then passed through a PD10 column (Sephadex G 25). The column was washed with PBS before adding the dialysed sample. The sample flow through was collected in 0.5ml fractions (~30 fractions).

2.2.15.2. ESTIMATION OF THE CCX IN THE CCX/BSA/EDC COMPLEX

Specific activity of ^3H -CCX = 6.5 mci/mg

used 2 μl /ml 3h-CCX = 1.5 μg ^3H -CCX/ml. 1 μg = 2.83×10^5 cpm/ μl .

20 μ moles of CCX = 13,360 μg

ratio cold/hot = 13,360/1.5 = 8960/1

after dialysis 3.35×10^3 cpm/ μl .

$3.35 \times 10^3 / 2.83 \times 10^5 = 0.0118 \mu\text{g}$; or 12 ng ^3H CCX

How much cold CCX present after dialysis?

$8906/1 \times X \mu\text{g} / 0.012 \mu\text{g} = 10.7 \mu\text{g}/\mu\text{l}$. = 10.7 mg/ml

2.2.15.3. INJECTION OF RABBITS WITH CCX/BSA CONJUGATE AND COLLECTION OF THE SERA.

Four rabbits were injected with the EDC linked CCX/BSA conjugate prepared in either complete (first injection) or incomplete Freund's adjuvant (400ul of Freund's adjuvant was mixed with 400ul of CCX/BSA conjugate and vortexed for 5 minutes to form an emulsion). Two rabbits were injected with 200ul of the conjugate (containing 1mg of CCX), and two rabbits with 100ul of the conjugate (containing 500 μg of CCX).

The rabbits were injected intramuscularly once every month, and bled 2 weeks later. Pre-immunisation and post-immunisation bleeds were collected, the blood allowed to clot, the blood clot cut and the sera collected. The serum was centrifuged at 1000g for 10 minutes to pellet debris and the clarified serum then pipetted out, and stored at -20°C prior to passage through an anti-BSA antibody binding column.

2.2.15.4. COUPLING OF THE CNBr-ACTIVATED SEPHAROSE BEADS WITH BOVINE SERUM ALBUMIN (BSA).

1. One gram of BSA was dissolved in 5ml of bicarbonate buffer (0.1M NaHCO_3 ; 0.5NaCl; pH 8.0).
2. Sepharose beads were placed in a sintered-glass filter funnel, and allowed to swell in 200ml of 1mM HCl solution (10 minutes), then washed with approximately 1 litre of 1mM HCl solution.
3. The beads were further washed with 200 ml of bicarbonate buffer.
4. The sepharose beads were then resuspended in 5ml BSA/bicarbonate buffer solution, followed by of 50ml of bicarbonate buffer to wash the beads into a 500ml beaker. The glass filter funnel was rinsed with a further 50ml bicarbonate buffer, and the wash was added to the 500ml beaker.
5. The BSA/sepharose beads were then dispensed into 50ml Falcon tubes, placed on a rotary mixer, and swirled for 2h at RT to assist coupling BSA to the sepharose beads.
6. The beads were then pooled by decanting the content of the tubes in to the sintered glass filter funnel, and the flow through volume measured .
7. After determining the optical density of the solution (by spectrophotometry), the coupling efficiency of BSA to the beads was measured as follows:
$$\text{mg of BSA bound to the column} = \text{O.D.} \times \text{volume of the flow through solution}$$
$$0.5 \times 100\text{ml} = 50\text{mg of BSA was not bound to the beads.}$$

Initially one gram of BSA was added to the beads therefore; $1000\text{mg} - 50\text{mg} = 950 \text{ mg.}$

Coupling efficiency = $950/1000 \times 100 = 90 \%$

- 2.2.15 8. The beads were then resuspended in 50 ml of ethanolamine and poured in to a clean 500ml beaker.
9. The sintered glass filter funnel was rinsed with 50ml of ethanolamine, and the rinse pooled with the beads in the 500ml beaker.
- 10 The content of the beaker was then dispensed in to 50ml falcon tubes, which were placed on a rotary mixer, and mixed for 2h at RT to block any remaining protein binding sites in the beads.
11. Ethanolamine was removed from the BSA/sepharose beads, which were then washed once with bicarbonate buffer, once with PBS, and stored at 4°C in PBS +0.01 % sodium azide.

2.2.15.5. REMOVAL OF ANTI-BSA ANTIBODY BY PASSAGE OF THE SERUM THROUGH AN ANTI-BSA ANTIBODY BINDING COLUMN.

A 20ml syringe barrel was plugged using a glass fibre filter paper disk (Whatman GF/C). BSA-conjugated sepharose beads in PBS were pipetted into the syringe barrel and allowed to form a column. Rabbit serum was then gently pipetted onto the top of the column and allowed to penetrate into the column, thereafter PBS was used to wash the serum through the column. Progress of the rabbit serum through the column could be monitored by observing the descent of the coloured band of the serum in the column. When the serum was close to the bottom the flow through the column was stopped, and the column barrel incubated for 2h at RT, after which flow through the column was resumed and serum samples were collected and stored at -20°C.

incubated at R.T in the dark for 10-15 minutes, after which the light absorbance at 405nm

2.2.15.6. DETECTION OF ANTI-CCX ANTIBODY IN THE RABBIT SERA BY ELISA.

In order to determine whether anti-CCX antibody was present in the rabbit serum an ELISA test was designed.

1. 50µl of CCX/KLH (containing 0.5µg CCX/well) was added to 96 well plates and incubated at 37°C overnight
2. The wells were washed 3 times with double-distilled water, and residual water removed by tapping the tray on absorbent paper.
3. The wells were then coated with 200ul of PBS+Tween20 (blocking buffer), and incubated at 37°C for 1h.
4. Plates were washed with double-distilled water three times and the residual water removed by tapping the plates on absorbent paper.
5. The immune or pre-immune serum as appropriate was then added to each well (50ul/well) and incubated at R.T for 1h.
6. Wells were again washed 3 times in double distilled water.
7. 50 ul of Horse-raddish peroxidase/ protein-A in PBS(1µg/ml) was added to each well, and the plates were incubated at R.T for 1h.
8. The plates were then washed 6 times in double distilled water.
9. 50µl of 2,2',-Azino-Bis (3-Ethylbenz-Thiazoline-6-sulfonic acid) diammonium salt (ABTS) at 0.5mg/ml (3µl of H₂O₂ was added/10ml of ABTS solution), in citrate/phosphate buffer (pH 4) was added per well. The plates covered, and

incubated at R.T in the dark for 10-15 minutes, after which the light absorbance at 405nm was read using a Titertek Multiskan spectrophotometer.

ANTIBODY- 1:50 dilution of anti-CCX antibody immune sera, or pre-immune in 100µl of 1% PBS 0.02% Tween20.

BLOCKING BUFFER (PBT)- 0.02% Tween20 in PBS.

CITRATE/PHOSPHATE BUFFER- 0.1M citric acid +0.1M Na_2PO_4 pH 4, dissolved in 100 ml of double distilled ultra-pure water.

SUBSTRATE-ABTS/ H_2O_2 - 0.5mg/ml of ABTS in citrate/phosphate buffer, 2ul of H_2O_2 was added for every 10ml of ABTS solution.

2.3. SOLUTIONS.

SOLUTIONS AND BUFFERS USED IN ELISA

ANTIBODY- 1:50 dilution of anti-CCX antibody immune sera, or pre-immune in

RESOLVING PBS 0.02% Tween20.

RESOLVING 18.15g

BLOCKING BUFFER (PBT)- 0.02% Tween20 in PBS.

CITRATE/PHOSPHATE BUFFER- 0.1M citric acid +0.1M Na₂ PO₄ pH. 4,

dissolved in 100 ml of double distilled ultra-pure water.

Bring up the volume to 100 ml with double-distilled ultra-pure water, pH. 4.

SUBSTRATE-ABTS/H₂O₂- 0.5mg/ml of ABTS in citrate/phosphate buffer, 3ul of

H₂O₂ was added for every 10ml of ABTS solution.

ultra-pure H₂O₂.

TANK BUFFER. TRIS

GLYCEROL

SDS

Bring up the volumes to 1 litre with DI water.

RESOLVING GELS

SOLUTIONS AND BUFFERS USED IN SDS POLYACRYLAMIDE GEL ELECTROPHORESIS.

Volume was brought up to 100 ml, solution was passed through a Whatman #1 filter

RESOLVING GEL BUFFER. TRIS HCl 18.15g

A- 10% POLYACRYLAMIDE (SDS 0.4g 60mls gels mini-gels)

Bring up the volume to 100 ml with double distilled ultra-pure water, pH. 8.9.

DOUBLE-DISTILLED ULTRA PURE H₂O 24.9ml 3 ml

30% A- 10% POLYACRYLAMIDE (SDS 0.4g 60mls gels mini-gels)

Bring up the volume to 100 ml with double-distilled ultra-pure water, pH. 6.7.

25% AMMONIUM PERSULFATE. (APS). 0.25g in 1ml of double-distilled ultra-pure H₂O.

B- 9% POLYACRYLAMIDE. 15ml

TANK BUFFER. TRIS 6.32g

DOUBLE-DISTILLED ULTRA PURE H₂O 27ml

30 % ACRYLAMIDE/0.8% BIS ACRYLAMIDE (SDS 1g 60mls gels mini-gels)

Bring up the volumes to 1 litre with DI water.

RESOLVING GELS

30% ACRYLAMIDE	BIS ACRYLAMIDE	0.6g
	ACRYLAMIDE	29.4g

Volume was brought up to 100 ml, solution was passed through a Whatman #1 filter paper.

A- 10% POLYACRYLAMIDE GEL.

	60mls gels	mini-gels
GEL BUFFER	15ml	2.5 ml
DOUBLE-DISTILLED ULTRA PURE -H₂O	24.9ml	2 ml
30% ACRYLAMIDE/0.8% BIS ACRYLAMIDE (SCOTLAB)	20.1ml	3.5 ml
25% APS	60µl	-
10% APS	-	100µl
TEMED	150µl	10µl

B- 9% POLYACRYLAMIDE.

GEL BUFFER	15ml
DOUBLE-DISTILLED ULTRA PURE H₂O	27ml
30 % ACRYLAMIDE/0.8% BIS ACRYLAMIDE (SCOTLAB)	18ml
25% APS	60µl
TEMED	150µl

SDS PAGE GEL FIX/STAIN SOLUTION-0.2% comassia blue R 250 in

C- 7.5 % SEPARATING MINIGEL.

GEL BUFFER	1.8 ml
DOUBLE DISTILLED ULTRA PURE WATER	3.6 ml
30% ACRYLAMIDE/0.8% BISACRYLAMIDE (SCOTLAB)	1.8 ml
10% APS	200µl
TEMED	10µl
SPACER GEL BUFFER	1ml

D- STACKING GEL FOR 60ML GELS.

STACKING- GEL BUFFER	5ml
DOUBLE DISTILLED ULTRA PURE WATER	12.34ml
30% ACRYLAMIDE	2.66ml
25% APS	60µl
TEMED	150µl

D- STACKING GEL FOR MINI-GEL SYSTEM.

STACKING GEL BUFFER	1.5 ml
DOUBLE DISTILLED ULTRA PURE WATER	3.5 ml
30% ACRYLAMIDE/0.8% BISACRYLAMIDE (SCOTLAB)	1 ml
10% APS	100µl
TEMED	10µl

SDS PAGE GEL FIX/STAIN SOLUTION- 0.2% commassie blue R 250 in methanol, water, acetic acid in the ratio of 50: 43: 7.

SDS PAGE GEL DESTAIN SOLUTION- methanol, water, acetic acid in the ratio of 50, 880, 70

BLOCKING BUFFER- 5% Goat serum in PBS.

SDS PAGE EXTRACTION BUFFER.

SPACER GEL BUFFER 1ml

25% SDS 0.8ml

β -MERCAPTOETHANOL 0.5ml

GLYCEROL 1ml

BROMOPHENOL BLUE 25 μ l

Extraction buffer was diluted 1+2 with double distilled ultra-pure water before use.

BLOTTING BUFFER (TOWBIN)

25mM Tris 15.1g

192mM Glycine 72g

20% Methanol 1000 ml

Made up to 5 litre with DI water, 1.5 ml of concentrated HCl was added pH. 8.3.

SOLUTION AND BUFFERS USED IN FIV IMMUNOSTAINING

IMMUNOSTAINING BUFFER-0.5% Goat serum (Goat serum) in PBS.

BLOCKING BUFFER- 5% Goat serum in PBS.

0.05M ACETATE BUFFER- 50mM sodium acetate, mixed with 50mM acetic acid pH. 4.8-5.0.

STAINING SOLUTION- 0.8% AMINO ETHYL CARBAZOLE (AEC) IN FORMAMIDE.

0.8% AEC	0.5ml
0.5 M ACETATE BUFFER	9.5ml
30% H ₂ O ₂	75µl

The mixture was filtered through a Whatman #1 filter paper.

SOLUTION AND BUFFERS USED IN IMMUNOPRECIPITATION.

IMMUNOPRECIPITATION EXTRACTION BUFFER

0.1 M TRIS pH.8.0

10% GLYCEROL

5% NP40

0.2mM PMSF

3. CHAPTER 0.5% SODIUM DEOXYCHOLATE

WASH BUFFER(LiCl).

0.6 M LiCl

0.1 M TRIS, pH. 8.0

1.4M Mercaptoethanol

Volume was brought up to 100 ml with double-distilled ultra-pure water.

3.1. INVESTIGATION OF THE EFFECTS OF TRITERPENOID COMPOUNDS ON UNINFECTED CELLS IN CULTURE

Dargan and Subak-Sharpe (1985), and Galt *et al* (1990) have classified different cell lines according to their tolerance to treatment with CCX concentrations up to, 300µM. The work described here extends these studies to include the cell lines used in this thesis. The effect of the triterpenoid compounds on percentage cell viability, and cell culture growth of feline (FeA, QN10S, and CrFX), and bovine (MDBK, BEL, and BEK) cells, was determined by trypan-blue dye exclusion tests, and enumeration of total cell number in cell cultures treated with increasing concentrations of CCX over several days.

3. CHAPTER THREE

RESULTS

3.1. INVESTIGATION OF THE EFFECTS OF TRITERPENOID COMPOUNDS ON UNINFECTED CELLS IN CULTURE

Dargan and Subak-Sharpe (1985), and Galt *et al* (1990) have classified different cell lines according to their tolerance to treatment with CCX concentrations up to, 300 μ M. The work described here extends these studies to include the cell lines used in this thesis. The effect of the triterpenoid compounds on percentage cell viability, and cell culture growth of feline (FeA, QN10S, and CrFK), and bovine (MDBK, BEL, and BEK) cells, was determined by trypan-blue dye exclusion tests, and enumeration of total cell number in cell cultures treated with increasing concentration of drugs over several days.

The effect of CCX treatment on the growth of CrFK cells is shown in Fig 7B. A replication index (R.I.= total number of cells at time of harvesting divided by the total number of cells at time zero) was used to measure growth. The replication indices for the drug-free control culture increased to 4.3 after 3 days incubation, reached a plateau value of 9.1 after 6 days and gave a value of 9.2 after 10 days incubation. Three days treatment with 25 μ M, 50 μ M, or 75 μ M CCX had no effect on the rate of cell culture growth. Treatment with 100 μ M CCX for 3 days resulted in a small impairment of cell growth (R.I. 3.72), while 150 μ M CCX significantly impaired cell culture growth (R.I. 1.6). Six days treatment with 25 μ M, or 50 μ M CCX had no significant effect on cell growth, but treatment with 75 μ M, 100 μ M, or 150 μ M CCX inhibited cell growth (R.I. 4.6, 4.4, and 1.1 respectively) and the R.I. range reached a plateau. After 10 days treatment

3.1.1. THE EFFECT OF CCX. OR CBX TREATMENT ON THE GROWTH AND PERCENTAGE VIABILITY OF CrFK CELLS IN CULTURE.

The effect of CCX treatment on CrFK cell cultures is shown in Fig 7A. The percentage cell viability of the drug-free control culture remained >90% for up to 8 days, but dropped to 80% after 10 days incubation. The cell viabilities of CrFK cultures treated with up to 100 μ M CCX for three days remained about 90%, but treatment with 150 μ M CCX reduced cell viability to 65%. After 6 days CCX treatment, cultures treated with 25 μ M, or 50 μ M CCX remained about 90% viable, while those treated with 75 μ M, 100 μ M, or 150 μ M were 83%, 77.1%, and 66% viable respectively. After 10 days treatment, cultures treated with 25 μ M, or 50 μ M CCX had percentage viabilities of 73.5%, and 18.8% respectively, while those cultures treated with higher drug concentrations were non-viable.

The effect of CCX treatment on the growth of CrFK cells is shown in Fig 7B. A replication index (R.I= total number of cells at time of harvesting divided by the total number of cells at time zero) was used to measure growth. The replication indices for the drug-free control culture increased to 4.3 after 3 days incubation, reached a plateau value of 9.1 after 6 days and gave a value of 9.2 after 10 days incubation. Three days treatment with 25 μ M, 50 μ M, or 75 μ M CCX had no effect on the rate of cell culture growth. Treatment with 100 μ M CCX for 3 days resulted in a small impairment of cell culture growth (R.I. 3.72), while 150 μ M CCX significantly impaired cell culture growth (R.I. 2.44). Six days treatment with 25 μ M, or 50 μ M CCX had no significant effect on the rate of cell growth, but treatment with 75 μ M, 100 μ M, or 150 μ M CCX inhibited culture growth (R.I's. 4.6, 4.4, and 1.1 respectively) and the R.I range reached a plateau. After 10 days treatment

with 25 μ M or 50 μ M CCX , there was a small decrease in the total number of cells (RI's, 6.28, and 6.81 respectively) possibly suggesting some lysis of cells in the culture. Only cellular debris was present in cultures treated with 75 μ M, 100 μ M, or 150 μ M CCX indicating complete lysis of the culture after 10 days treatment.

The duration of the FIV replication cycle in CrFK cells is 3 days. It was, therefore, of particular interest to determine the effect of the drugs on uninfected CrFK cell cultures during this period. The data in Figures 7A and 7B show that, there was little evidence for CCX-induced cytotoxic effect when CrFK cells were treated with up to 100 μ M CCX for 3 days.

The effect of CBX on CrFK cell viability is shown in Fig 8A. The percentage cell viability of the drug-free control cultures remained at greater than 90% throughout the 10 days incubation period. Three days treatment with drug concentrations up to 150 μ M CBX had no significant effect on cell viabilities (greater or equal to 90%). Six days treatment with up to 100 μ M CBX also had no significant effect on cell viabilities, but 6 days treatment with 150 μ M CBX resulted in a small decrease in cell viability to 86.5%. After 10 days treatment with 25 μ M, or 50 μ M CBX there was no significant effect on cell viability, but 10 days treatment with 75 μ M, 100 μ M, or 150 μ M CBX resulted in progressive reduction in cell viability to 73.8%, 69.5%, and 0% respectively.

The effect of CBX treatment on the growth of CrFK cells is shown in Fig 8B. The replication indices for the drug-free control culture increased to a value of 4.4 after 3 days incubation, reached a plateau value of 9.1 after 6 days incubation, and fell to 6.8 after 10

days incubation indicating some lysis of cells in over-confluent drug-free cultures at late times after incubation.

Three days treatment with up to 100μM CBX had no significant effect on the rate of cell growth, but treatment with 150μM CBX impaired replication of the cell culture (RI. 2.6). Six days treatment with 25μM, 50μM, 75μM, 100μM, or 150μM CBX resulted in a decrease in RI values to 8.0, 7.5, 6.0, and 4.0 respectively compared to the drug-free control. Treatment with CBX concentrations greater than 25μM for 6 days resulted in the RI curve either reaching a plateau or declining. After 10 days treatment there was little difference between the control RI value and the RI values obtained in the cultures treated with up to 75μM of CBX. Treatment with 100μM CBX gave an RI value of 5.1, while only cellular debris was present in cultures treated with 150μM CBX for 10 days, suggesting complete lysis of the culture.

The data in Figures 8A and 8B show that there was no evidence for cytotoxicity after 3 days treatment with up to 100μM CBX.

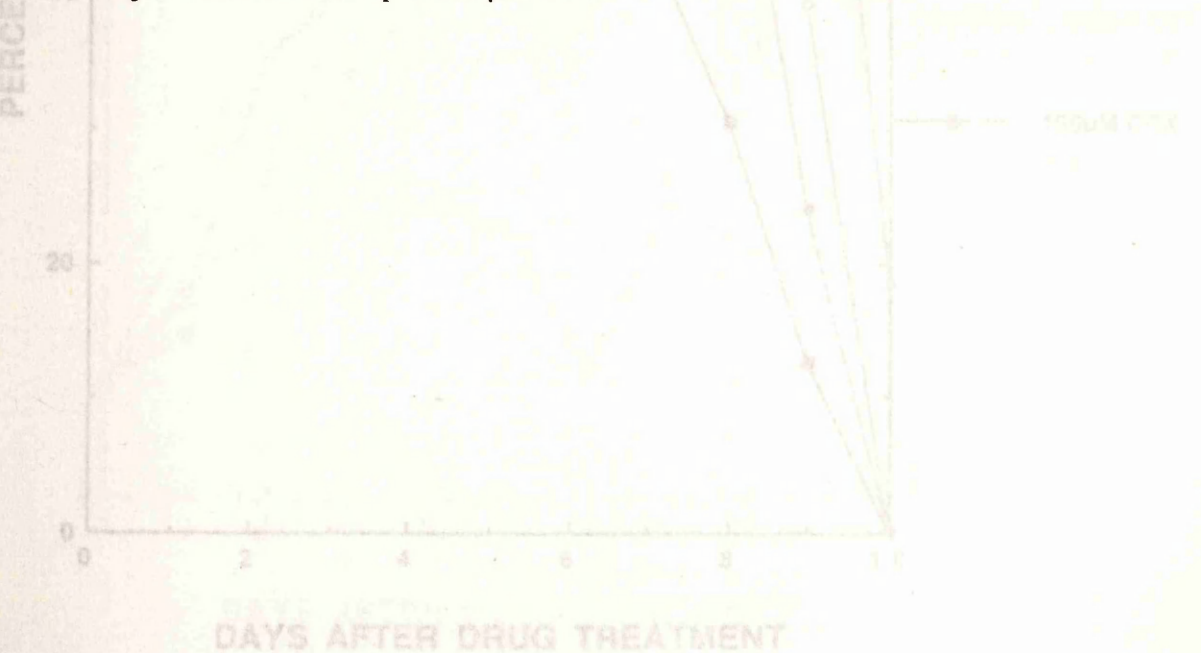


Figure. 7A
 THE EFFECT OF CCX ON CrFK CELL VIABILITY.

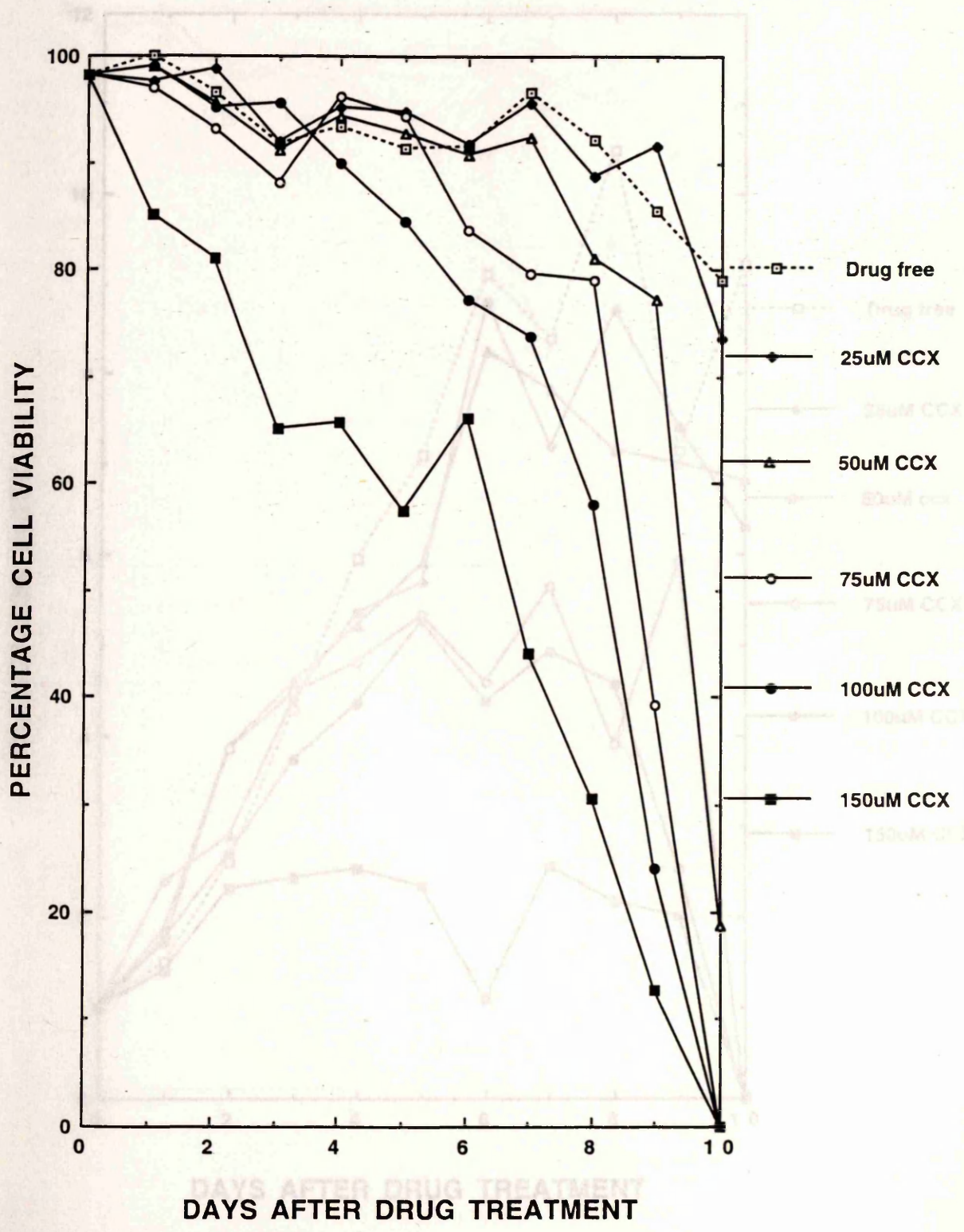


Figure. 7B
THE EFFECT OF CCX ON CrFK CELL GROWTH
(REPLICATION INDEX).

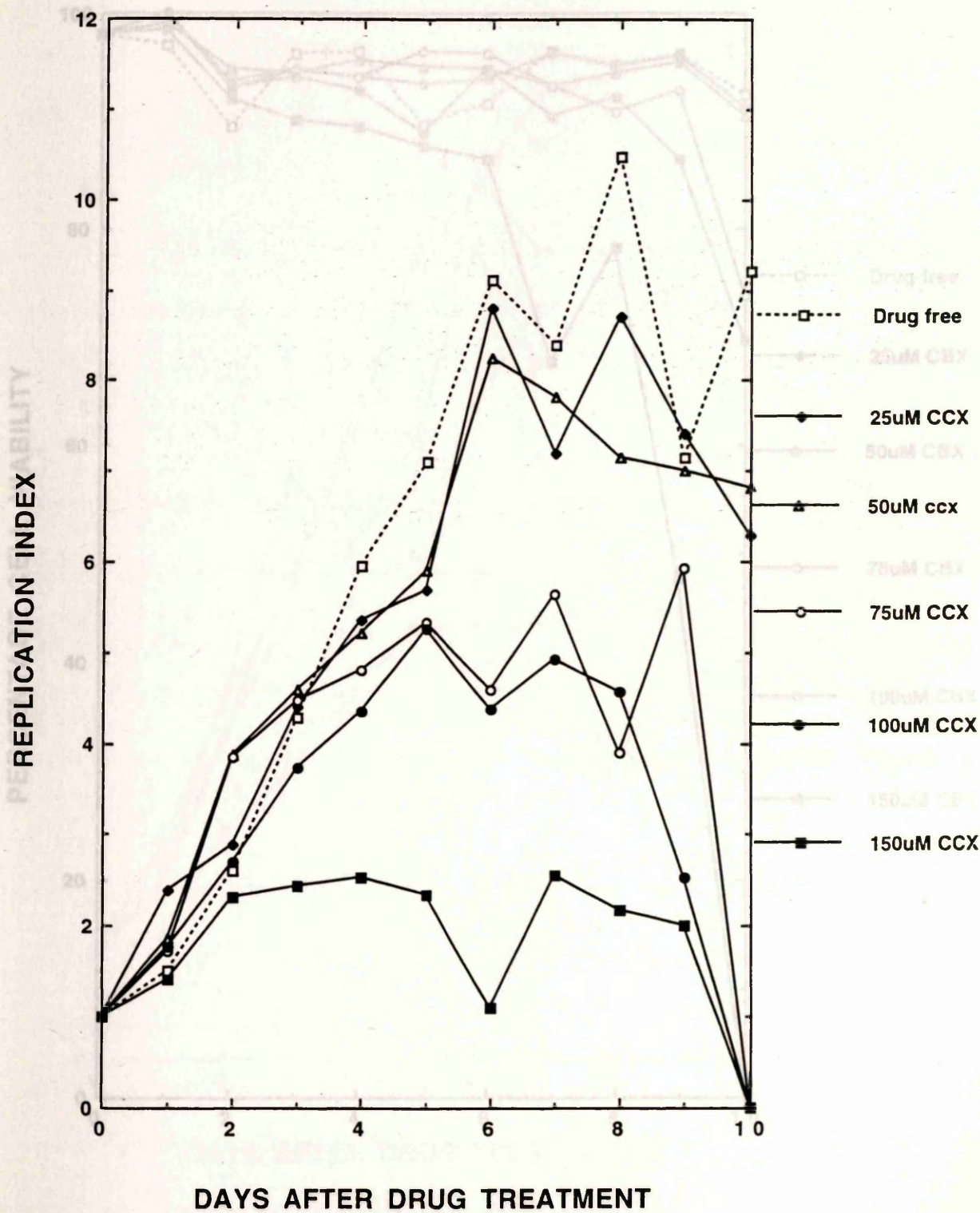


Figure. 8B

THE EFFECT OF CBX ON CrFK CELL GROWTH

THE EFFECT OF CBX ON CrFK CELL VIABILITY.

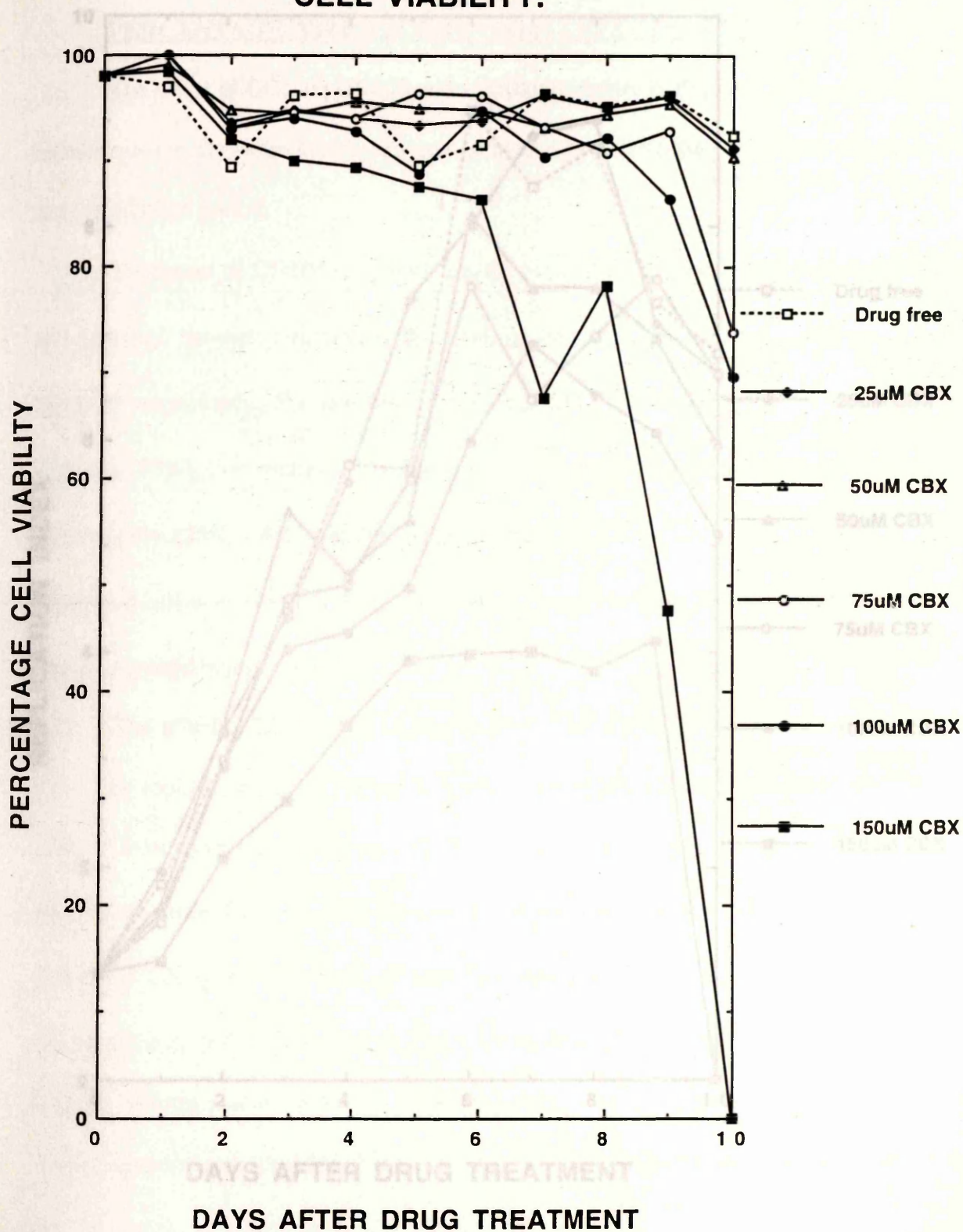
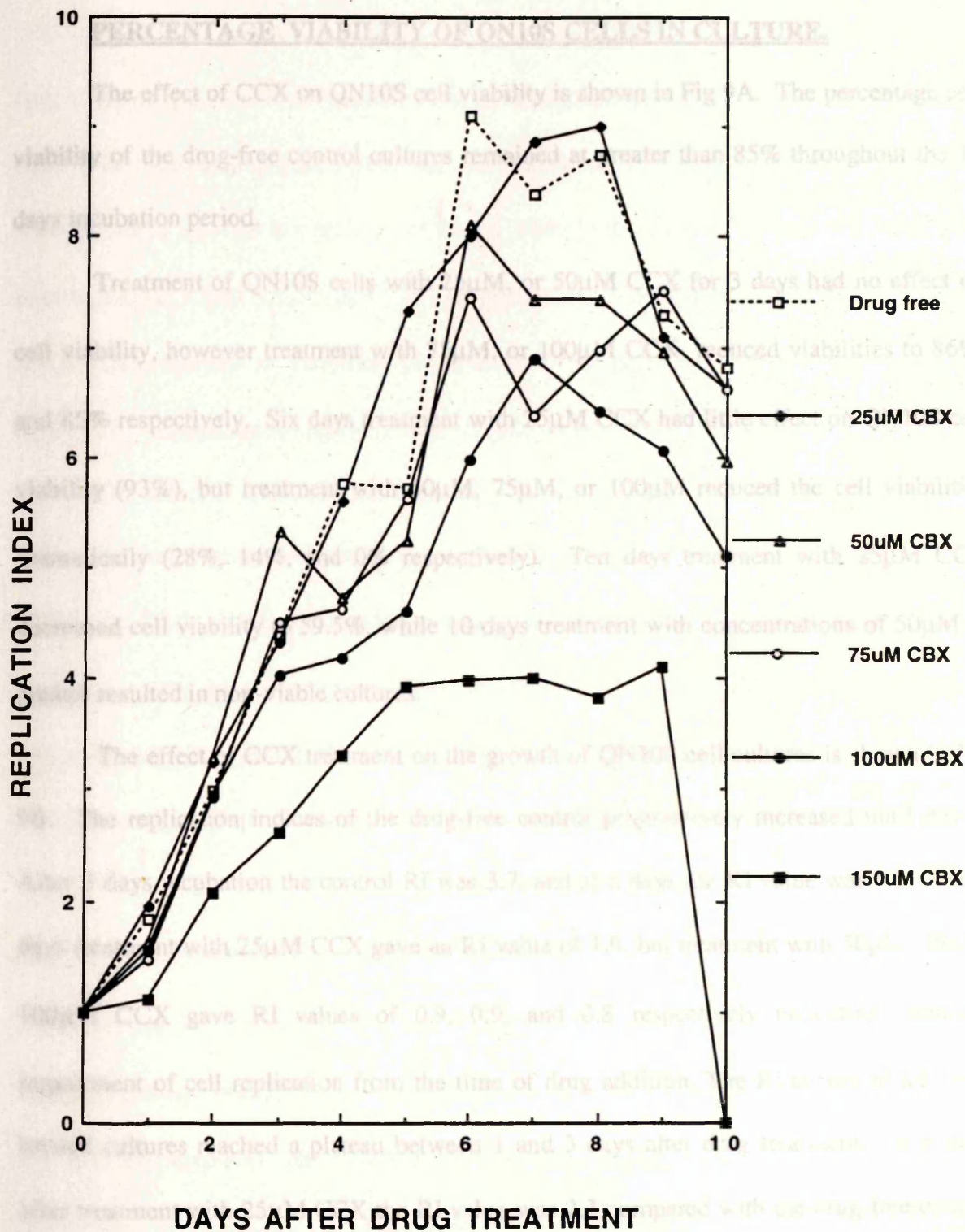


Figure. 8B
THE EFFECT OF CBX ON CrFK CELL GROWTH
(REPLICATION INDEX).



3.1.2. THE EFFECT OF CCX, CBX, OR GL TREATMENT ON GROWTH AND PERCENTAGE VIABILITY OF QN10S CELLS IN CULTURE.

The effect of CCX on QN10S cell viability is shown in Fig 9A. The percentage cell viability of the drug-free control cultures remained at greater than 85% throughout the 10 days incubation period.

Treatment of QN10S cells with 25 μ M, or 50 μ M CCX for 3 days had no effect on cell viability, however treatment with 75 μ M, or 100 μ M CCX reduced viabilities to 86%, and 85% respectively. Six days treatment with 25 μ M CCX had little effect on QN10S cell viability (93%), but treatment with 50 μ M, 75 μ M, or 100 μ M reduced the cell viabilities dramatically (28%, 14%, and 0% respectively). Ten days treatment with 25 μ M CCX decreased cell viability to 59.5%, while 10 days treatment with concentrations of 50 μ M or greater resulted in non-viable cultures.

The effect of CCX treatment on the growth of QN10S cell cultures is shown in Fig 9B. The replication indices of the drug-free control progressively increased until day 7. After 3 days incubation the control RI was 3.7, and at 6 days the RI value was 5.0. Three days treatment with 25 μ M CCX gave an RI value of 1.9, but treatment with 50 μ M, 75 μ M, 100 μ M CCX gave RI values of 0.9, 0.9, and 0.8 respectively indicating complete impairment of cell replication from the time of drug addition. The RI curves of all drug-treated cultures reached a plateau between 1 and 3 days after drug treatment. At 6 days after treatment with 25 μ M CCX the RI value was 2.3 compared with the drug-free control

value at 8.3. After 10 days treatment with 25 μ M CCX the RI value was 1.35, while treatment with higher drug concentrations resulted in lysis of the culture.

The effect of CBX treatment on the viability of QN10S cell cultures is shown in Fig 10A. The drug-free control was the same as that given in Fig 9A. Three days treatment with up to 100 μ M CBX gave percentage cell viabilities of greater or equal to 90%, while 6 days treatment resulted in viabilities of 94.5%, 88%, 76%, and 62% in cultures treated with 25 μ M, 50 μ M, 75 μ M, or 100 μ M CBX respectively. Ten days treatment with 25 μ M, or 50 μ M CBX resulted in viabilities of 84%, and 79% respectively, while treatment with higher drug concentrations resulted in non-viable cultures after 10 days incubation.

The effect of CBX treatment on the growth of QN10S cell cultures is shown in Fig 10B. The drug-free control was that described in Fig 9B. After three days treatment with 25 μ M, or 50 μ M CBX there was no significant difference in the rate of cell growth compared to the drug-free control. Treatment with 75 μ M, or 100 μ M CBX for three days, however, impaired cell replication slightly (RI's values of 1.6, and 1.3 respectively compared with 3.7 for control). The RI values for 50 μ M, and 75 μ M CBX reached a plateau value after 3 to 4 days drug treatment, indicating that the culture had stopped growing. Cultures treated with 100 μ M CBX failed to grow from the time of drug addition. There was no detectable effect on cell culture growth after 6 days treatment with 25 μ M CBX. Treatment with 50 μ M, or 75 μ M, CBX, however, impaired cell replication (RI's. 2.1, and 1.57, respectively). After 10 days treatment with 25 μ M, 50 μ M, or 75 μ M, CBX, the RI values were 5.1, 1.84, and 1.2, respectively, while the drug-free control RI value was 8.22.

The effect of GL treatment on the percentage cell viability of QN10S cells in culture is shown in Fig 11A. The percentage viability of the drug-free control was greater than 85% throughout the 9 days incubation period. Three days treatment with GL concentrations up to 5000 μ M had no significant effect on QN10S cell viability (greater than 85% viability). After six days treatment with up to 2000 μ M GL there was no significant effect on cell viability, but the percentage viabilities of cultures treated with 2500 μ M, 3000 μ M, 4000 μ M or 5000 μ M decreased to 61%, 38%, 38%, and 16% respectively. There was no detectable effect on the viability of QN10S cell cultures treated with 500 μ M, or 1000 μ M GL for 9 days, but treatment with 1500 μ M, 2000 μ M, 2500 μ M, 3000 μ M, 4000 μ M, or 5000 μ M resulted in viabilities of 43.5%, 18%, 18%, 16%, 0%, and 0% respectively.

The effect of GL treatment on the growth of QN10S cell cultures is shown in Fig 11B. The replication indices for the drug-free control culture increased progressively until 5 days incubation (RI. 8.6), and then progressively declined to 5.45 after 9 days. Three days treatment with 500 μ M, 1000 μ M, 1500 μ M, 2000 μ M, 2500 μ M, 3000 μ M, 4000 μ M, or 5000 μ M GL resulted in progressive inhibition of cell culture growth; the RI. values reached were 4.6, 4.2, 3.6, 2.7, 3.3, 1.7, 1.16, and 1.7 respectively. The RI curves for cultures treated with 1000 μ M, 1500 μ M, 2000 μ M, 2500 μ M, 3000 μ M, 4000 μ M, or 5000 μ M GL reached near plateau values or declined after 2 to 4 days incubation (RI values; 4.57, 4.2, 3.0, 1.6, 1.3, 1.15, and 0.8 respectively). After 6 days treatment only the cultures treated with 500 μ M GL provided evidence for cell culture growth (RI. 6.43) compared with the 3 day RI value of 4.6. After 9 days incubation there was no significant difference

between the RI value obtained from the culture treated with 500μM GL and the drug-free control culture, while cultures treated for 9 days with 1000μM, 1500μM, 2000μM, 2500μM, 3000μM, 4000μM, or 5000μM GL resulted in RI values of 5.71, 4.68, 2.17, 1.49, 1.81, 1.60, and 1.03 respectively.

The data in Figures 9, 10, and 11 indicate that QN10S cells tolerate treatment with 25μM CCX, 50μM CBX, or 1000μM GL for up to 3 days. Although the QN10S cell line is sensitive to triterpenoid-induced cytotoxicity, it was used only for titration of the FeLV-A virus. During virus titration any residual drug would have been diluted below these critical concentrations. Furthermore the exposure of QN10S cells to the drugs was limited to the 2h virus absorption period, after which the cell layers were washed.

In conclusion, residual drug, carried over in virus titration samples is unlikely to induce cytotoxicity in the QN10S indicator cell line.

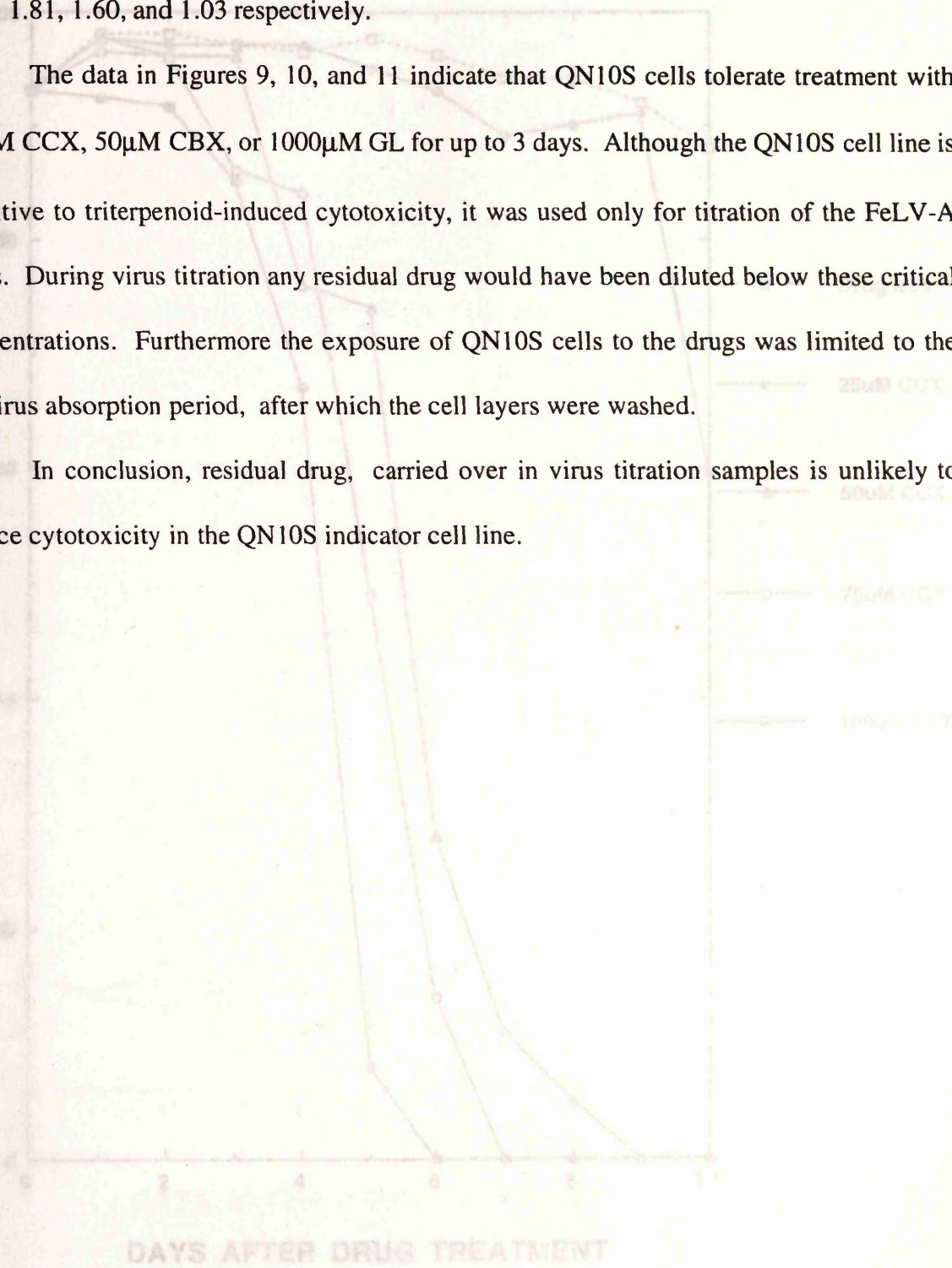


Figure. 9B

THE EFFECT OF CCX ON QN10S CELL GROWTH

Figure. 9A
THE EFFECT OF CCX ON QN10S
CELL VIABILITY.

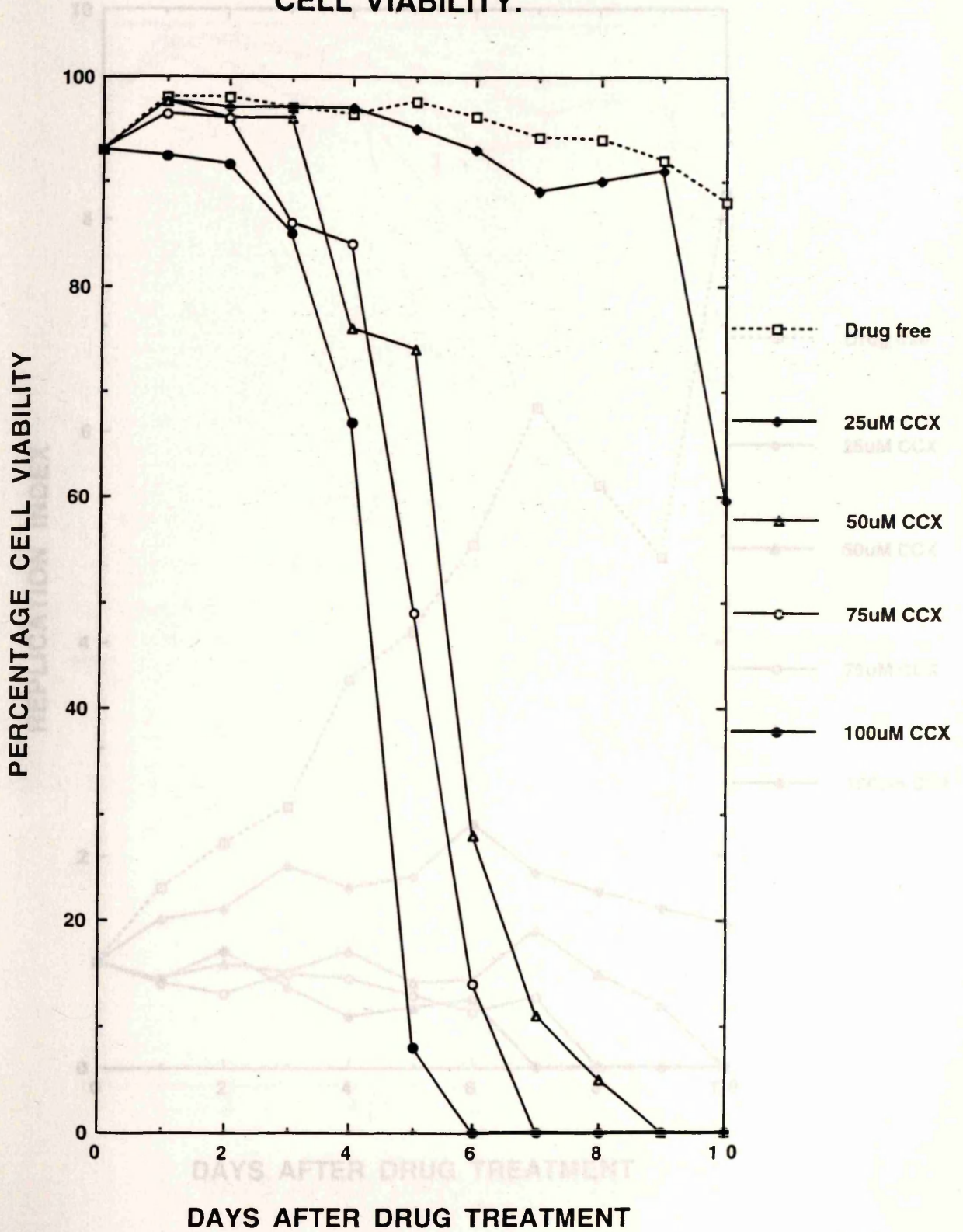


Figure. 9B
THE EFFECT OF CCX ON QN10S CELL GROWTH
(REPLICATION INDEX).

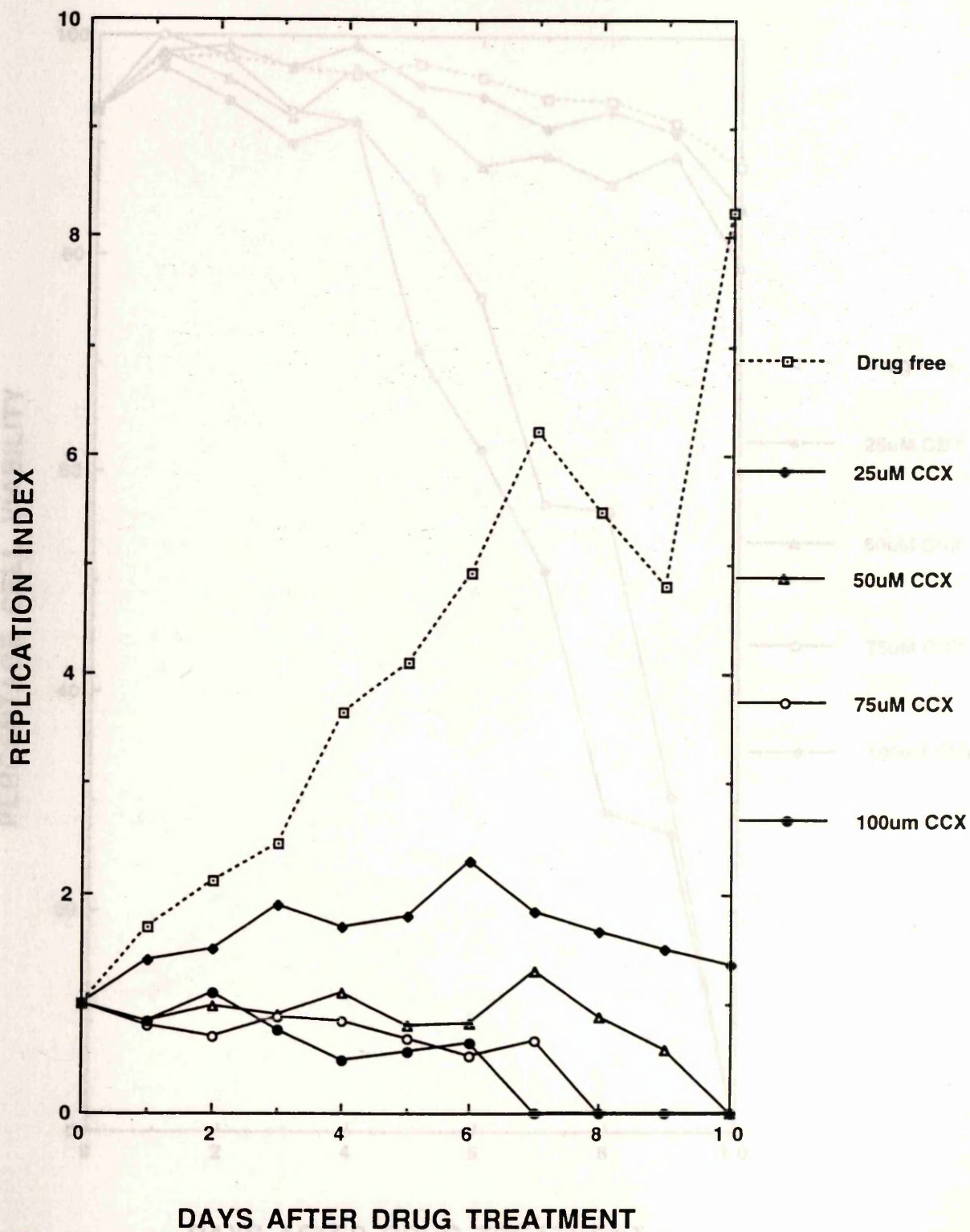


Figure. 10A
THE EFFECT OF CBX ON QN10S CELL VIABILITY.

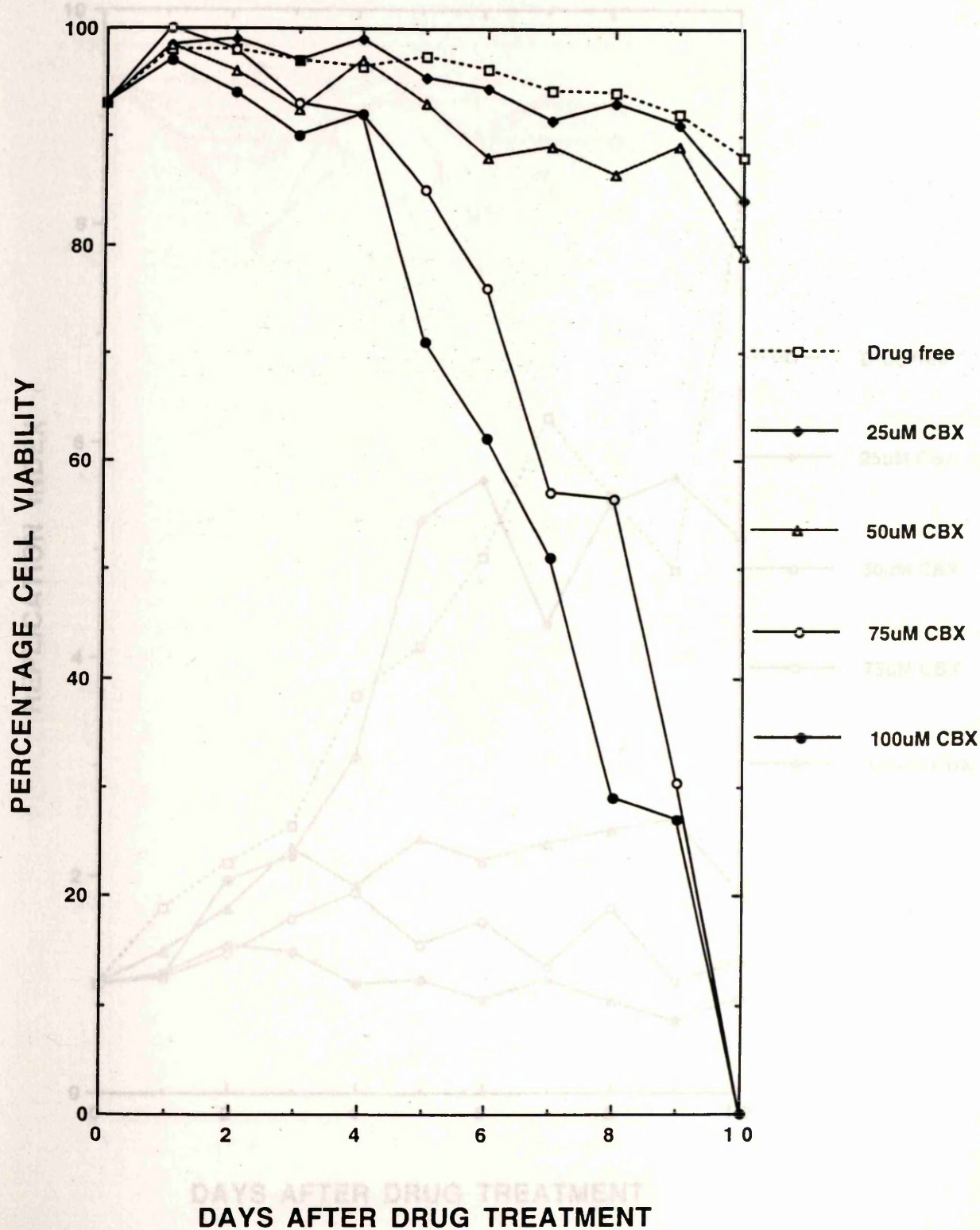


Figure. 10B
THE EFFECT OF CBX ON QN10S CELL GROWTH
(REPLICATION INDEX).

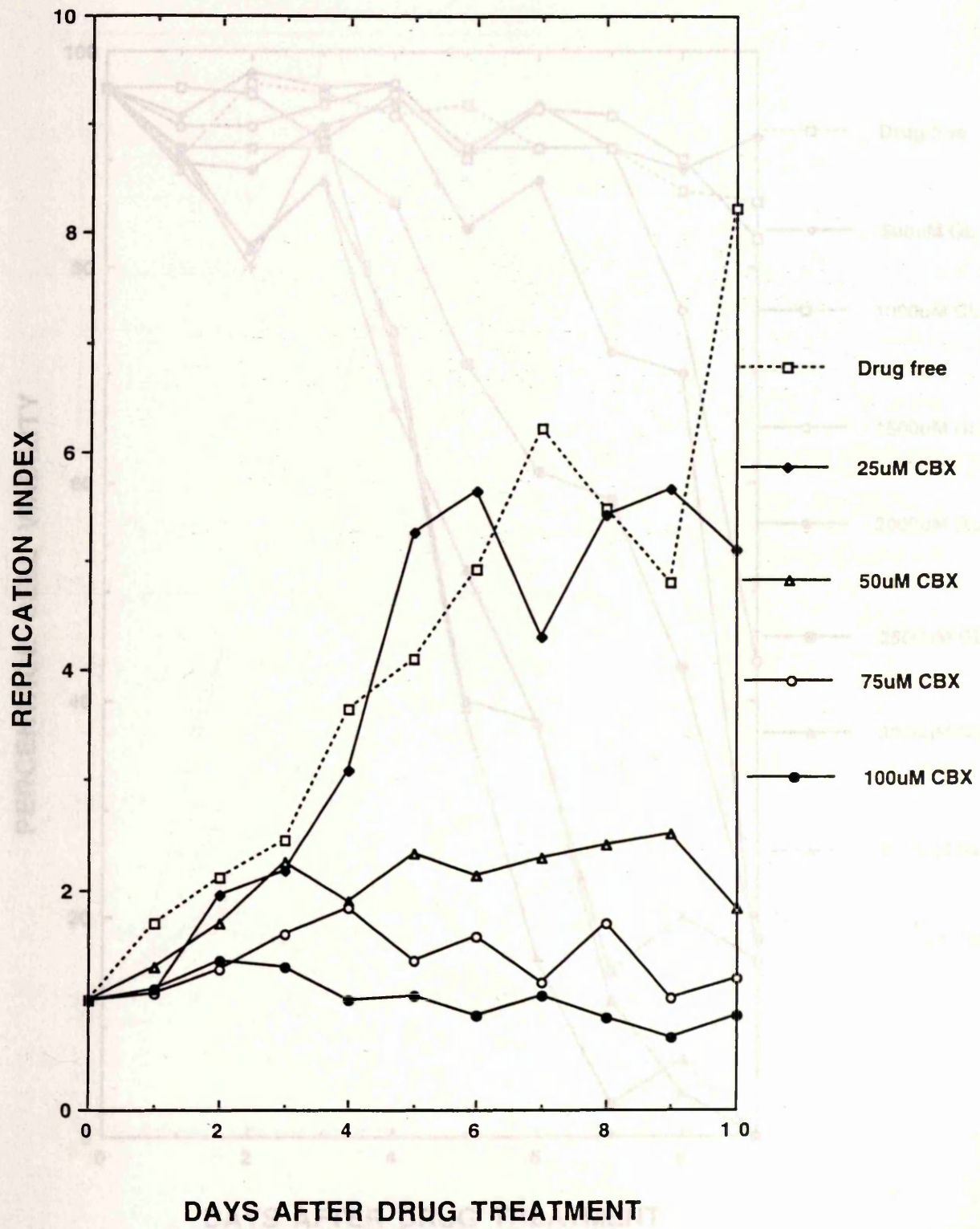


Figure. 11A
 THE EFFECT OF GL ON QN10S
 CELL VIABILITY.

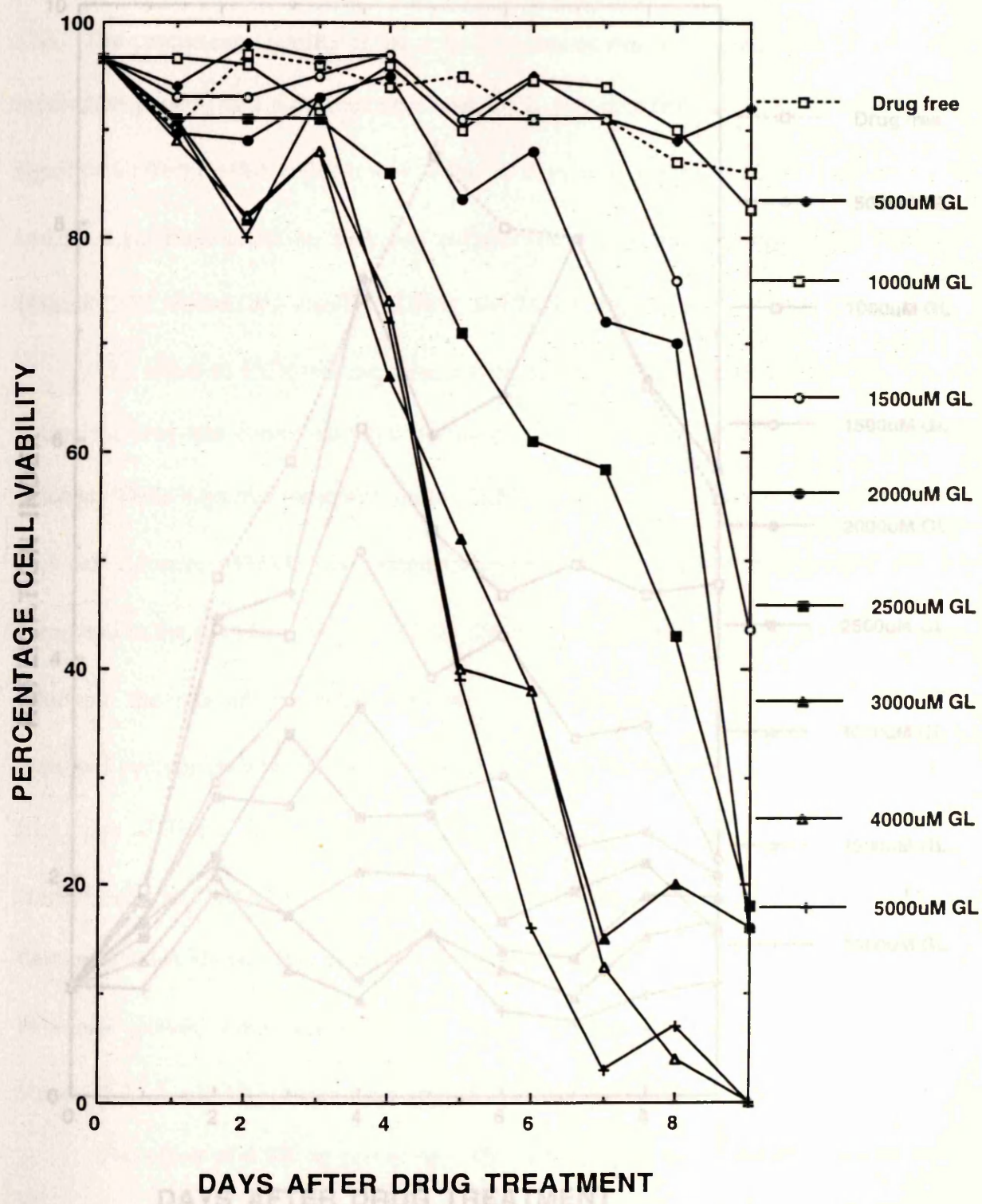
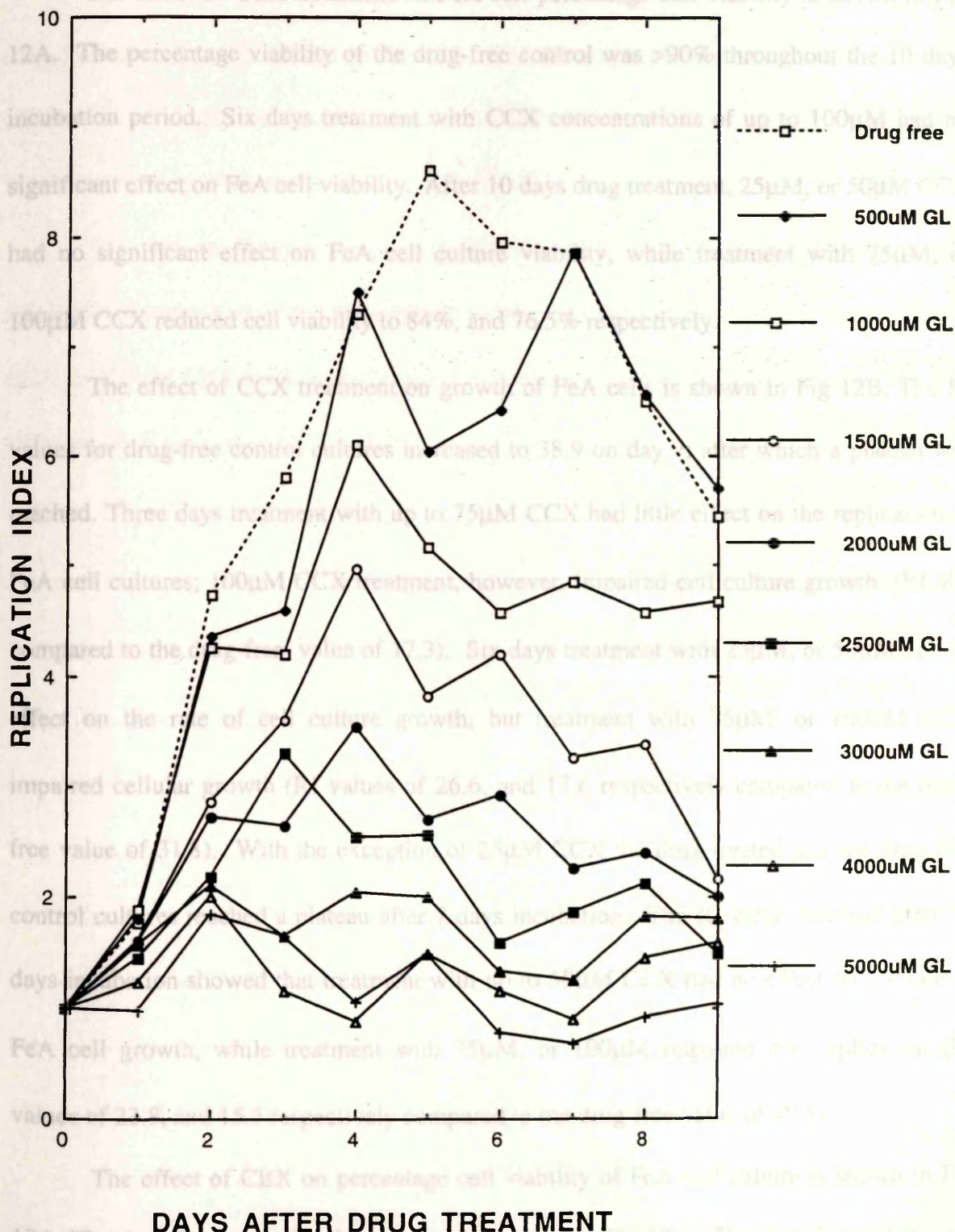


Figure. 11B
THE EFFECT OF GL ON QN10S CELL GROWTH
(REPLICATION INDEX).



3.1.3. THE EFFECT OF CCX . OR CBX TREATMENT ON GROWTH AND PERCENTAGE VIABILITY OF FeA CELLS IN CULTURE.

The effect of CCX treatment on FeA cell percentage cell viability is shown in Fig 12A. The percentage viability of the drug-free control was >90% throughout the 10 days incubation period. Six days treatment with CCX concentrations of up to 100 μ M had no significant effect on FeA cell viability. After 10 days drug treatment, 25 μ M, or 50 μ M CCX had no significant effect on FeA cell culture viability, while treatment with 75 μ M, or 100 μ M CCX reduced cell viability to 84%, and 76.5% respectively.

The effect of CCX treatment on growth of FeA cells is shown in Fig 12B. The RI values for drug-free control cultures increased to 38.9 on day 7, after which a plateau was reached. Three days treatment with up to 75 μ M CCX had little effect on the replication of FeA cell cultures; 100 μ M CCX treatment, however, impaired cell culture growth. (RI. 8.7 compared to the drug-free value of 17.3). Six days treatment with 25 μ M, or 50 μ M had no effect on the rate of cell culture growth, but treatment with 75 μ M, or 100 μ M CCX impaired cellular growth (RI values of 26.6, and 17.6 respectively compared to the drug-free value of 31.8). With the exception of 25 μ M CCX the drug-treated and the drug-free control cultures reached a plateau after 7 days incubation. The RI value obtained after 10 days incubation showed that treatment with up to 50 μ M CCX had no effect on the rate of FeA cell growth, while treatment with 75 μ M, or 100 μ M impaired cell replication (RI values of 23.8, and 15.5 respectively compared to the drug-free value of 40.5)

The effect of CBX on percentage cell viability of FeA cell culture is shown in Fig 13A. The drug-free control is the same as that shown in Fig 12A. The data shows that even

10 days treatment with up to 100μM CBX had no effect on the viability of FeA cell cultures.

The effect of CBX on the growth of FeA cell cultures is shown in Fig 13B. The drug-free control is that shown in Fig 12B. The RI curves obtained for drug-treated cell cultures closely approximates that of the drug-free control, indicating that FeA cell growth is not impaired by treatment with up to 100μM CBX.

The data in Figures 12 (A and B) and 13 (A and B) show that significant cytotoxicity was not encountered when FeA cell cultures were treated with up to 100μM CCX or 100μM CBX for 3 days (the maximum duration of antiviral experiments). Clearly, CBX was less toxic, than CCX to FeA cell cultures.

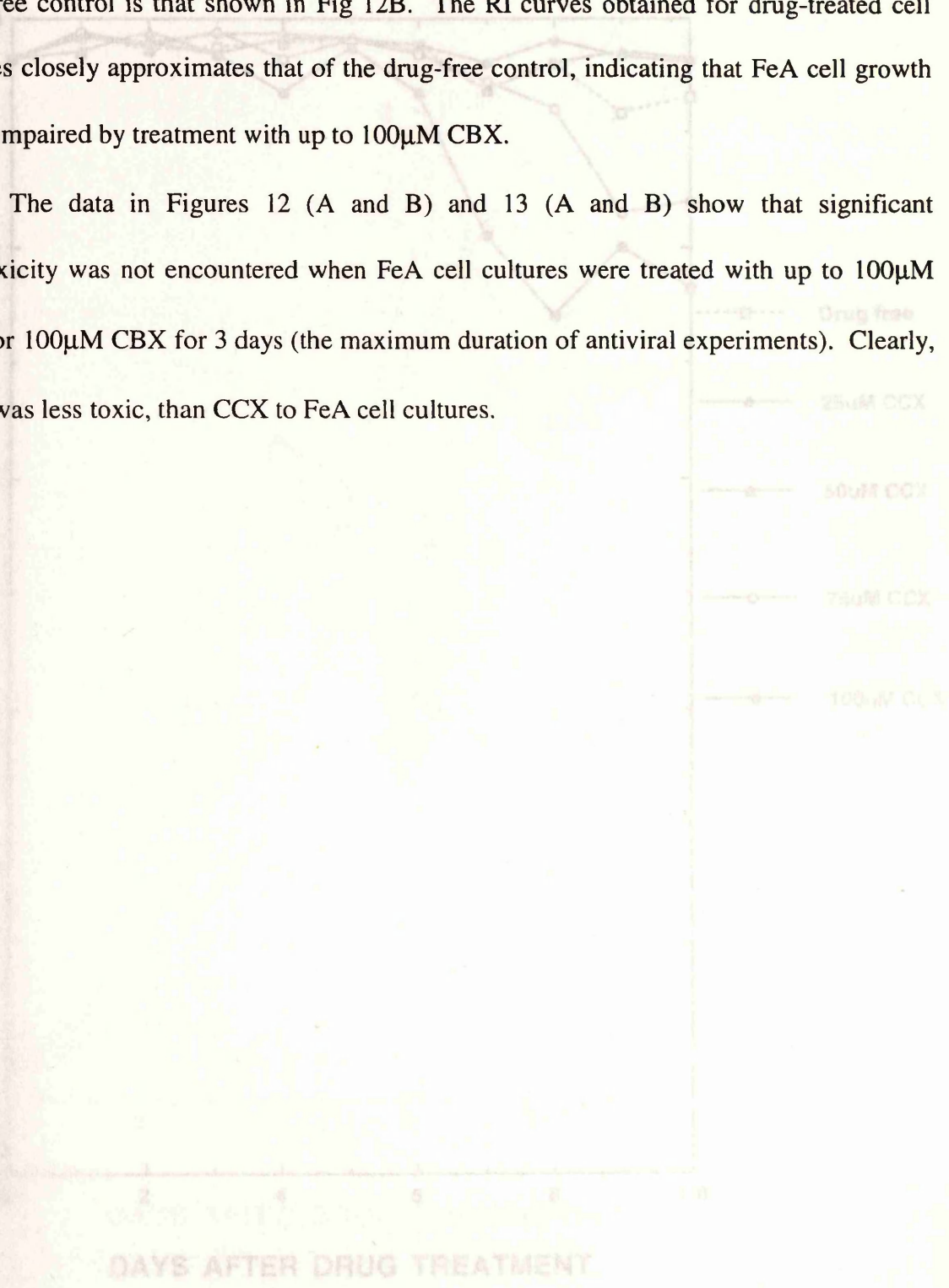


Figure. 12B

THE EFFECT OF CCX ON FeA CELL GROWTH

Figure. 12A
THE EFFECT OF CCX ON FeA
CELL VIABILITY.

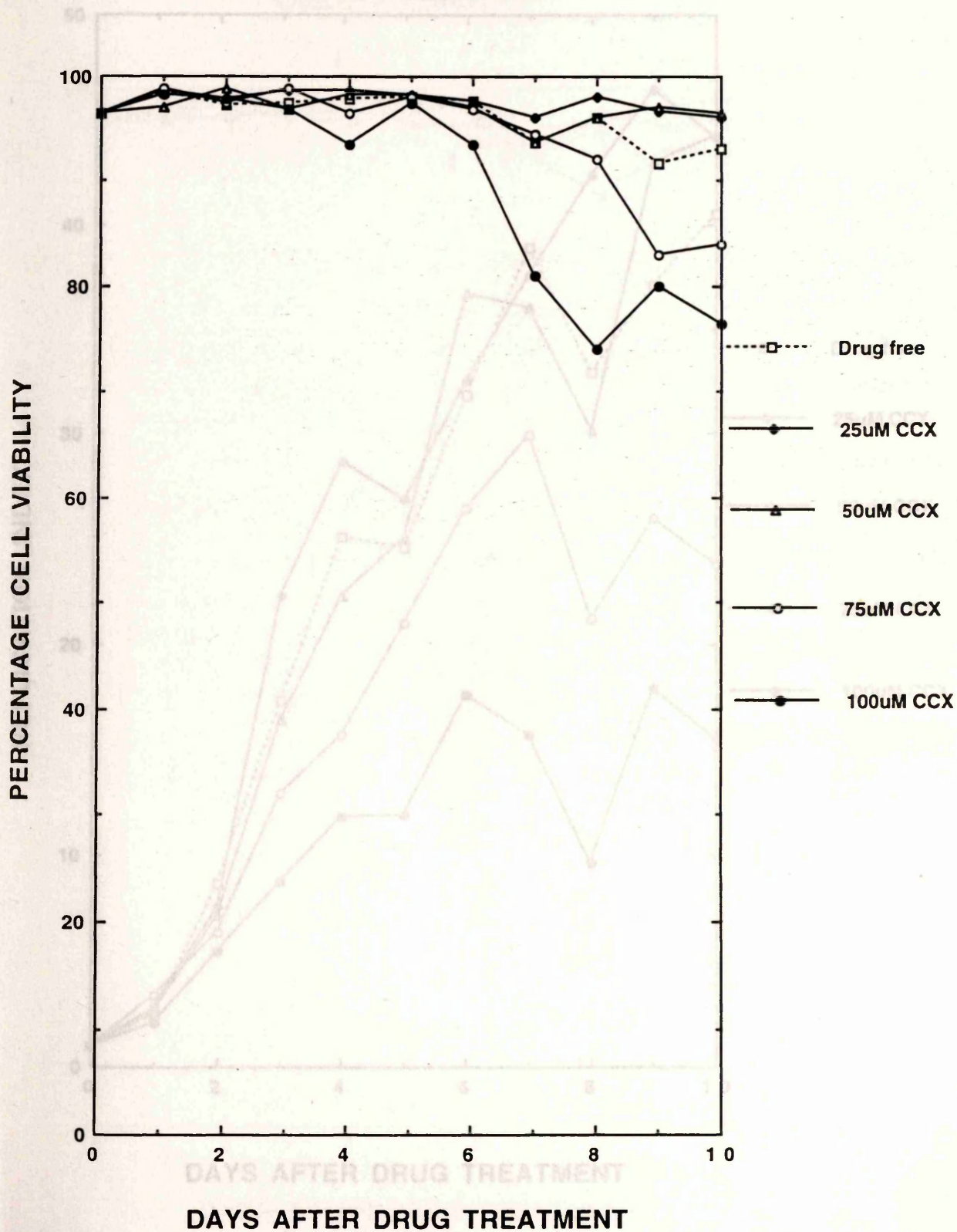


Figure. 12B
THE EFFECT OF CCX ON FeA CELL GROWTH
(REPLICATION INDEX).

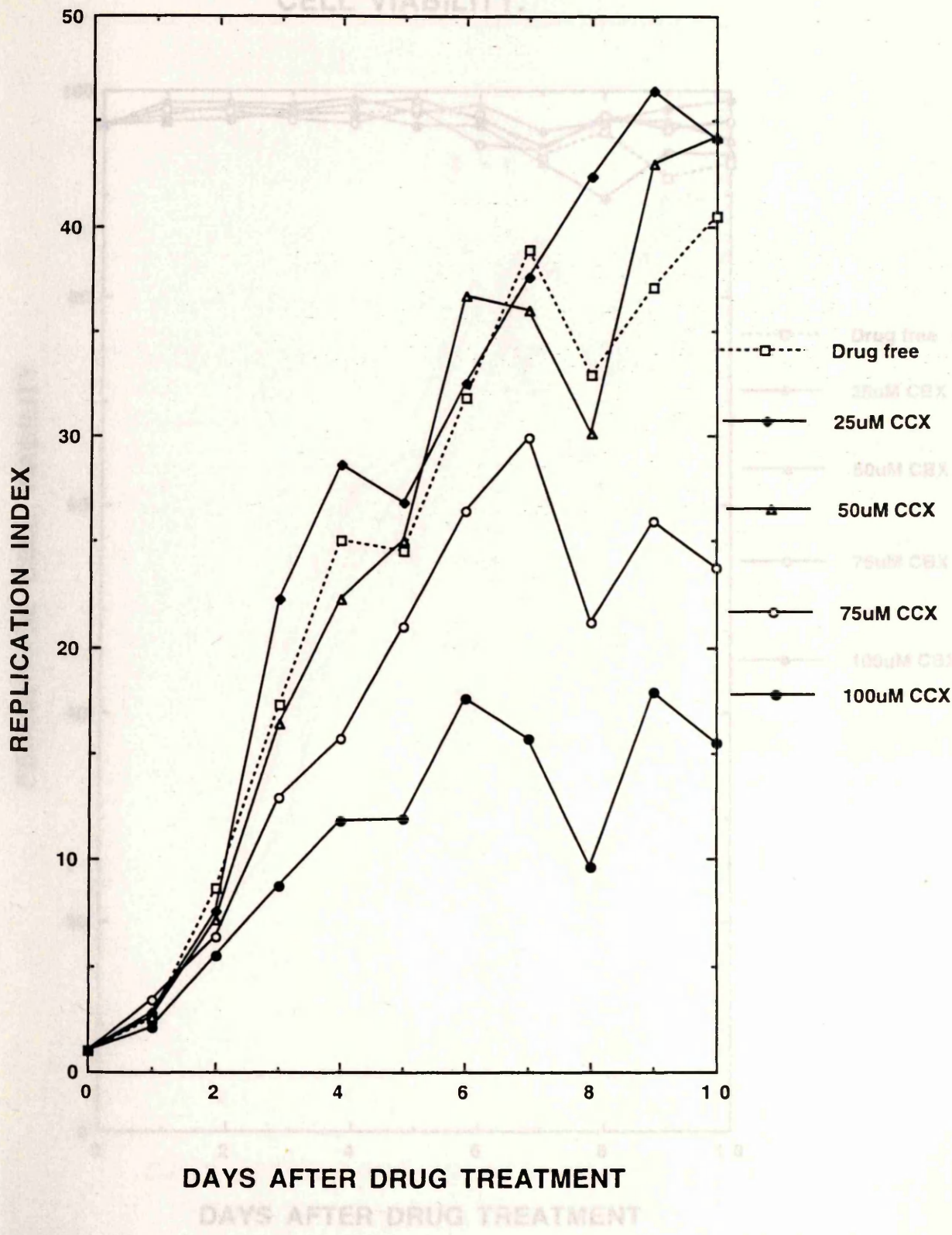


Figure 13A

THE EFFECT OF CBX ON FeA CELL VIABILITY.

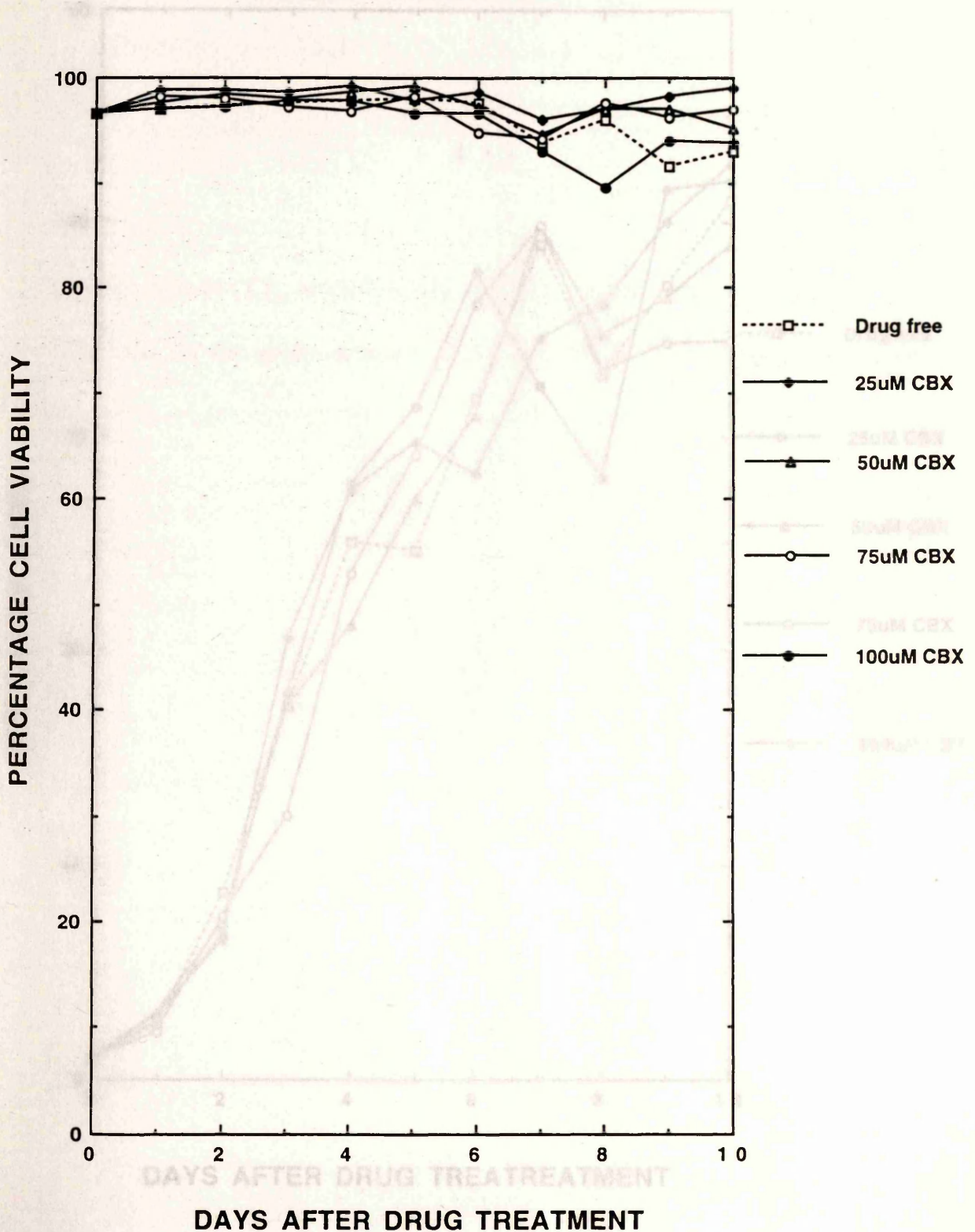
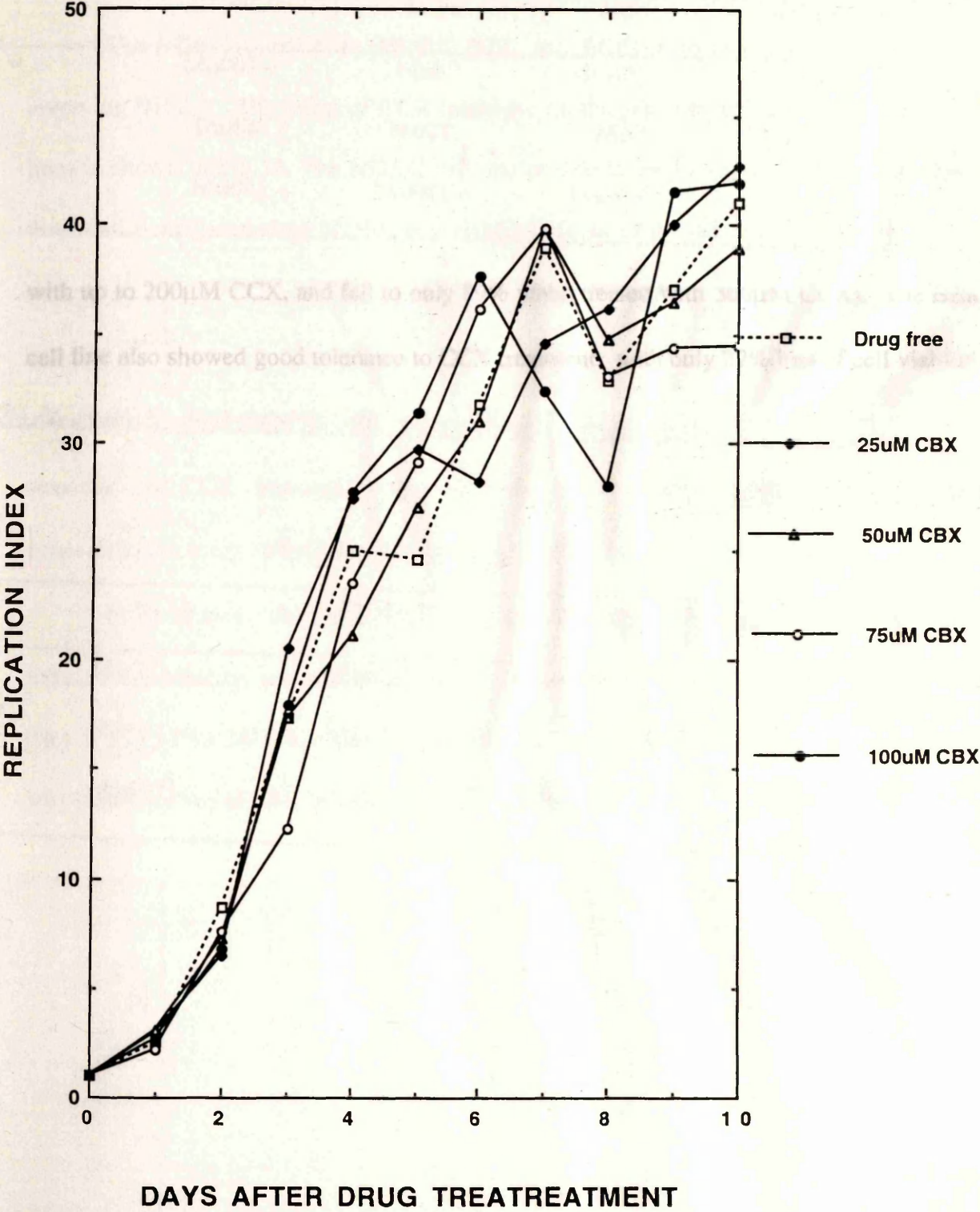


Figure. 13B
THE EFFECT OF CBX ON FeA CELL GROWTH
(REPLICATION INDEX).



Concentration of CCX inhibiting cell replication by 10%, 25%, or 50% (IC10, IC25, IC50) at 48h after drug treatment.

Cell Line	IC10	IC25	IC50
FeA	19uM	70uM	> 100uM
QN10S	8uM	22uM	48uM
CrFK	136uM	> 150uM	> 150uM

Concentration of CBX inhibiting cell replication by 10%, 25%, or 50% (IC10, IC25, IC50) at 48h after drug treatment.

Cell Line	IC10	IC25	IC50
FeA	9uM	24uM	> 100uM
QN10S	30uM	59uM	> 100uM
CrFK	115uM	140uM	> 150uM

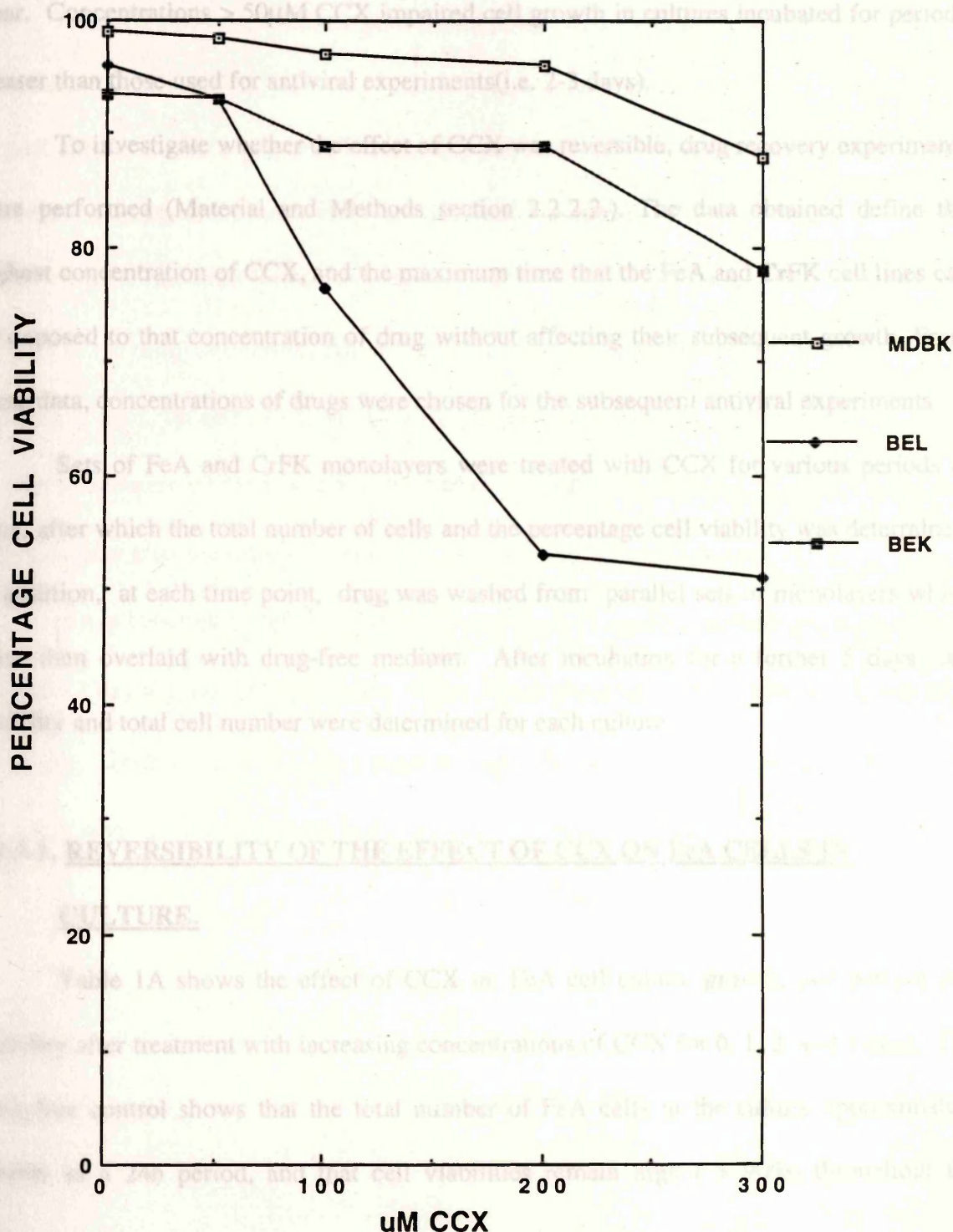
3.1.3. THE EFFECT OF 24h CCX TREATMENT ON THE VIABILITIES OF MADIN-DARBY BOVINE KIDNEY (MDBK), BOVINE EMBRYO LUNG (BEL), AND BOVINE EMBRYO KIDNEY (BEK) CELLS IN CULTURE.

The following cell lines (MDBK, BEL, and BEK) were available for experiments involving BHV-1. The effect of CCX treatment on the percentage viabilities of these cell lines is shown in Fig 14. The MDBK cell line proved to be the most CCX-tolerant of the three cell lines (percentage MDBK cell viability remained greater than 95%, when treated with up to 200 μ M CCX, and fell to only 89% when treated with 300 μ M CCX). The BEK cell line also showed good tolerance to CCX treatment, with only 22% loss of cell viability when treated with 300 μ M CCX. The BEL cell cultures, however, proved the most sensitive to CCX treatment and cell viability fell rapidly with increasing drug concentrations (only 50% of the cells were viable after 24h treatment with 300 μ M).

In conclusion, the MDBK cells displayed the highest level of resistance to CCX-induced cytotoxicity, and were selected for subsequent antiviral studies with BHV-1. The data in Fig 14 for MDBK cells is consistent with the earlier findings of Galt *et al* (1990) who classified the MDBK cells as CCX-resistant.

3.1.5. RECOVERY OF CCX-TREATED FeA AND CrFK CELL CULTURES

Figure. 14
THE EFFECT OF 24 h TREATMENT WITH CCX
ON MDBK, BEL, AND BEK CELL VIABILITY.



3.1.5. RECOVERY OF CCX-TREATED FeA AND CrFK CELL CULTURES

AFTER DRUG REMOVAL.

While even 6 days treatment of FeA and CrFK cell cultures with up to 100 μ M CBX did not give rise to significant cytotoxicity, the situation with CCX treatment was not so clear. Concentrations > 50 μ M CCX impaired cell growth in cultures incubated for periods greater than those used for antiviral experiments(i.e. 2-3 days).

To investigate whether the effect of CCX was reversible, drug recovery experiments were performed (Material and Methods section 2.2.2.2.). The data obtained define the highest concentration of CCX, and the maximum time that the FeA and CrFK cell lines can be exposed to that concentration of drug without affecting their subsequent growth. From these data, concentrations of drugs were chosen for the subsequent antiviral experiments.

Sets of FeA and CrFK monolayers were treated with CCX for various periods of time, after which the total number of cells and the percentage cell viability was determined. In addition, at each time point, drug was washed from parallel sets of monolayers which were then overlaid with drug-free medium. After incubation for a further 5 days, cell viability and total cell number were determined for each culture.

3.1.5.1. REVERSIBILITY OF THE EFFECT OF CCX ON FeA CELLS IN CULTURE.

Table 1A shows the effect of CCX on FeA cell culture growth, and percent cell viability after treatment with increasing concentrations of CCX for 0, 1, 2, and 3 days. The drug-free control shows that the total number of FeA cells in the culture approximately double in a 24h period, and that cell viabilities remain high (> 90%) throughout the

experiment. The data show that cell viability was not impaired until cultures were treated with 200µM CCX or greater for 2 days or longer. Cell culture growth was significantly impaired as a result of 3 days treatment with 100µM, 2 days treatment with 150µM, or 1 day treatment with 200µM CCX or greater.

Table 1B shows cell culture growth and percentage viability in parallel FeA cultures 5 days after CCX removal, following 1, 2, or 3 days treatment with the drug. The drug-free control culture shows a pattern of decreasing rises in total cell number multipliers due to increasing confluence of the monolayer with time and contact inhibition of the cells,. Cell cultures which had been treated with up to 100µM CCX for 3 days replicated with similar kinetics to the drug-free control culture. Cell cultures, which had been treated with 150µM CCX for 2 days, failed to replicate after drug release, even though cell viability was 95%. Similarly, cultures which had been treated with 200µM or 300µM CCX for only 1 day failed to grow after the drug was removed, even though cell viability was > 80%.

It was concluded that the effect of treatment of FeA cell cultures with up to 150µM CCX for 1 day was partially reversible, while 3 days treatment with 100µM CCX was fully reversible. Thus 100µM CCX treatment of FeA cell cultures for 3 days, or 150µM CCX for 1 day were taken as the upper limits for subsequent antiviral studies.

REVERSIBILITY OF THE EFFECT OF CCX ON FeA CELLS IN CULTURE.

Table 1 A. The effect of CCX on FeA cell number and percentage viability before drug removal.

uM CCX	DAYS OF DRUG TREATMENT			
	ZERO	ONE	TWO	THREE
0	6 (97)**	11 (97)	33 (99)	100 (96)
50	10.3 (97)	16 (96)	29 (98)	78 (96)
100	5.0 (94)	13 (95)	28 (97)	43 (93)
150	0.7 (97)	10 (97)	10 (96)	13 (89)
200	0.6 (87)	8 (98)	6 (84)	4 (64)
300		8 (96)	4 (58)	4 (16)

* Numbers indicate the percent of total cell number in each culture, relative to the total number of cells in the drug free control at 3 days.

** Percent cell viability in each culture.

REVERSIBILITY OF THE EFFECT OF CCX ON FeA CELLS IN CULTURE.

Table 1 B. The effect of CCX removal on FeA cell number and cell viability, after 1, 2, or 3 days drug treatment.

DAYS OF DRUG TREATMENT			
UM CCX	ONE	TWO	THREE
0	14.1 (97)**	5.2 (96)	2.7 (98)
50	14.6 (98)	5.1 (96)	3.0 (97)
100	10.3 (97)	5.8 (98)	3.9 (97)
150	5.0 (94)	1.2 (95)	1.2 (93)
200	0.7 (97)	0.6 (86)	0.6 (88)
300	0.6 (81)	0.6 (63)	0.1 (0)

* Numbers are multipliers indicating the increase in total cell number during the 5 day recovery period, and are relative to the numbers observed in individual cultures (table 1 A) after 1, 2, or 3 days drug treatment

** Percentage cell viability.

3.1.5.2. REVERSIBILITY OF THE EFFECT OF CCX ON CrFK CELLS IN CULTURE.

Table 2A shows the effect of CCX treatment on CrFK cell culture growth and cell culture viability after treatment with increasing concentrations of CCX for 0, 1, 2, or 3 days.

The drug-free control culture shows that total CrFK cell number approximately doubled in 24h, and that the percentage viability in the experiment ranged from 87% to 90%. The data show that cell viability was not affected until cultures were treated with 200 μ M CCX or greater for 2 days (76%), or 300 μ M CCX for 1 day (17%). Surprisingly, CrFK cell culture growth was impaired (RI. value of 19.6 compared to drug-free value of 28.2), after 1 day treatment with 100 μ M CCX in this experiment, but as shown in Fig 7B CrFK cell culture replication is not usually impaired under these conditions.

Table 2B shows cell culture growth and percentage viability in parallel CrFK cell cultures 5 days after CCX removal, following 1, 2, or 3 days treatment with the drug. The drug-free control culture shows a trend toward decreasing rises in total cell number multipliers with time. The percent viabilities of the drug-free culture remain > 80% throughout the experiment. Cell cultures which had been treated with 50 μ M CCX for 3 days, or 100 μ M CCX for 2 days grew either more rapidly, or at about the same rate as, the drug-free control cultures. Treatment with 150 μ M CCX, or greater, however resulted in partial (150 μ M for 1 day), or irreversible impairment of cell culture growth, even though cell culture viabilities generally remained at about 70%.

REVERSIBILITY OF THE EFFECT OF CCX ON CrFK CELLS IN CULTURE.

In conclusion, impairment of cell culture growth caused by treatment of CrFK cultures with up to 100µM CCX for 2 days is reversible. Thus 100µM CCX treatment of CrFK cell cultures for 2 days was taken as the upper limits for subsequent antiviral studies.

3.1.6. PREPARATION OF AN ANTI-CCX ANTIBODY GENERATED IN RABBITS AND ITS DETECTION BY ELISA TESTS.

In order to investigate the intracellular location of CCX molecules in uninfected cells, an attempt was made to generate an antibody against CCX in New Zeland White rabbits. Since CCX is a relatively small molecule (Mw. 668.8) EDC was used to mediate an amino-carboxy coupling to link CCX via its COONa grouping to amino groups on BSA (Material and Methods section).

Four New Zeland white rabbits (age 6 weeks) were injected intramuscularly with the CCX/BSA conjugate in the presence of Freund's adjuvant. Rabbits 1 and 2 were injected with the mixture at final concentration of 500µg CCX/rabbit, while rabbits 3 and 4 each were injected with the mixture containing 1mg of CCX. Prior to immunisation, rabbits were bled to collect pre-immune sera (PI), which was processed, then stored at 20°C. The rabbits were injected with the CCX mixture once a month, and bled two weeks later, a total of 7 bleeds were taken, and after processing the immune sera were also stored at -20°C. To enrich the immune sera for potential anti-CCX antibodies, the serum samples were then passed through a BSA sepharose beads to remove antibodies directed against BSA proteins.

In order to detect anti-CCX antibody present in the serum samples, representative serum samples from bleeds 1, 2, 3, 4, 5, 6, and 7 from the four rabbits and their pre-immune

REVERSIBILITY OF THE EFFECT OF CCX ON CrFK CELLS IN CULTURE.

Table 2 A. The effect of CCX on CrFK cell number and percentage viability before drug removal.

DAYS OF DRUG TREATMENT				
uM CCX	ZERO	ONE	TWO	THREE
0	18* (94.5)**	28 (90)	79 (90)	100 (87)
50	50	27 (96.5)	70 (87)	100 (82)
100	100	20 (93)	32 (91)	63 (86)
150	150	20 (86)	39 (80)	40 (89)
200	200	18 (95)	18 (76)	24 (86)
300	300	17(17)	21 (9)	9 (22)

* Numbers indicate the percent of total cell numbers in each culture, relative to the total cell number in the drug free control at 3 days.

** Percent cell viability in each culture.

ND. Cell number not determined, no viable cells observed.

REVERSIBILITY OF THE EFFECT OF CCX ON CrFK CELLS IN CULTURE.

Table 2 B. The effect of CCX removal, on CrFK cell number and cell viability after 1, 2, or 3 days drug treatment.

uM CCX	DAYS OF DRUG TREATMENT		
	ONE	TWO	THREE
0	9 (87)**	2 (83)	3 (82)
50	7 (85)	2 (67)	2 (77)
100	7 (89)	3 (78)	0.8 (88)
150	3 (79)	0.62 (68)	0.8 (69)
200	2 (68)	1 (69)	0.8 (57)
300	ND	ND	ND

* Numbers are multipliers indicating the increase in total cell number during the 5 day recovery period, and are relative to the numbers observed in individual cultures (table 1B) after 1, 2, or 3 days drug treatment.

** Percentage cell viability

ND. Cell number not determined, no viable cells observed.

TABLE 3. DETECTION OF AN ANTI-CCX ANTIBODY: ELISA

sera were investigated using an ELISA protocol. CCX alone, or conjugated to KLH was added directly to wells in a 96 well microtitre tray, while BSA, protein-A, and ABTS controls were added to other wells. Protein-A and ABTS constitute the ELISA detection system and give a colour change in the presence of antigen + antibody reaction.

Table3 shows the values obtained for wells containing the controls. The antigen (CCX/KLH) alone gave negative or extremely low values, while the test serum in the absence of antigen gave a negative value. Various test sera in the absence of antigen, but in the presence BSA gave negative values. Test sera in wells coated with CCX alone (not bound to KLH) gave either negative values or low positive values.

The PI, from the four rabbits gave negative values for CCX/KLH coated wells, while the immune sera gave positive values ranging from 0.378 to 1.727 (most of the values were grouped around one) (table 4). The data suggest that a specific anti-CCX antibody was detected by ELISA.

Much time and effort was spent in attempting to detect CCX molecules in drug-treated uninfected cells by immunofluorescence. Several problems were encountered. The PI sera strongly and non-specifically labelled the cytoplasm of drug-treated MDBK and control cells (titrating out at 1/3200) (data not shown). In contrast the immune sera strongly labelled the nucleus of drug-treated cells and less strongly the nucleus of control cells. This nuclear fluorescence was finally determined to be non-specific (titrating out at 1/400) (data not shown).

TABLE 3. DETECTION OF AN-ANTI-CCX ANTIBODY: ELISA CONTROLS.

SERA	UNBOUND CCX	CCX/KLH	BSA	PROTEIN A	ABTS	VALUES
B1/S3	-	-	+	+	+	-0.059
B2/S3	-	-	+	+	+	-0.169
B7/S3	-	-	+	+	+	-0.137
PI/S3	-	-	+	+	+	-0.028
B1/S3	+	-	-	+	+	-0.038
B2/S3	+	-	-	+	+	+0.14
B6/S3	+	-	-	+	+	-0.003
PI 1	+	-	-	+	+	+0.089
PI 2	+	-	-	+	+	-0.063
PI 3	+	-	-	+	+	-0.084

B-BLEED
S-SAMPLE
PI-PREIMMUNE SAMPLE

+ = presence in control experiment
- = absence in control experiment.

Values

+ = positive ELISA OD reading
- = negative ELISA OD reading

Numbers do not indicate strength of the antibody response, only its presence.

TABLE 4. DETECTION OF AN ANTI-CCX ANTIBODY: ELISA TEST RESULTS

SAMPLE	PRE-IMMUNE	BLEED 1	BLEED 2	BLEED 3	BLEED 4	BLEED 5	BLEED 6	BLEED 7
RABBIT 1	-	+	+	+	+	+	+	+
RABBIT 2	-	+	+	+	+	+	+	+
RABBIT 3	-	+	+	+	+	+	+	+
RABBIT 4	-	+	+	+	+	+	+	+

- + = positive values on ELISA OD readings, indicating the presence of the anti-CCX antibody.
- = negative values on ELISA OD readings, indicating the absence of the anti-CCX antibody.

3.1.7. INVESTIGATION OF THE EFFECT OF CCX ON MDBK AND CrFK CELLS. USING GOLGI-SPECIFIC ANTI-P58 ANTIBODY.

Previous studies have suggested that CCX impaired glycosylation of HSV, VSV, and SFV glycoproteins (Dargan et al., 1992). The effect of CCX treatment on the Golgi apparatus of uninfected CrFK or MDBK cells was investigated using an antibody directed against the trans-Golgi p58 protein in immunofluorescence experiments. P58 is localised in the trans cisternae of the Golgi apparatus where it binds to microtubules. Presence of p58 in the Golgi is thought to provide anchorage site for microtubules on the outer surface of the Golgi (Bloom, G., and Brashear, T., J. 1989)

MDBK and CrFK cells were seeded at 2×10^5 cells/well on glass coverslips in a 24 well Nunc tray. Following overnight incubation at 37°C, the growth medium was replaced with either drug-free medium or medium containing CCX. After 5h incubation at 37°C the cells were processed for immunofluorescence studies using the anti-p58 antibody as a tag for the Golgi apparatus, and FITC-conjugated anti-mouse antibody to detect the p58

Representative micrographs giving the results of these experiments are shown in Figures 15 and 16. In the absence of CCX, p58 gave perinuclear (Golgi) fluorescence with both CrFK and MDBK cells (Figs 15A, and 16A respectively). In the case of MDBK cells, however, there was also some diffuse cytoplasmic fluorescence (Fig 16A). After treatment of either cell line with 300µM CCX for 5h there was a reduction in perinuclear (Golgi) and cytoplasmic (MDBK cells only) fluorescence (Figs 15B and 16B). In the MDBK cells there was a concomitant manifestation of punctate fluorescence in the nucleus of cells (16B). Punctate nuclear fluorescence was also observed in CrFK cells but only in small percentage

of cells (15B). The reduction in the p58 tagging of the Golgi suggests an effect of the drug on that organelle.

Commercially available antibody directed against another Golgi marker protein (β -cop) failed to tag any structure in MDBK or CrFK cells even in the absence of CCX.

FIGURE 15.

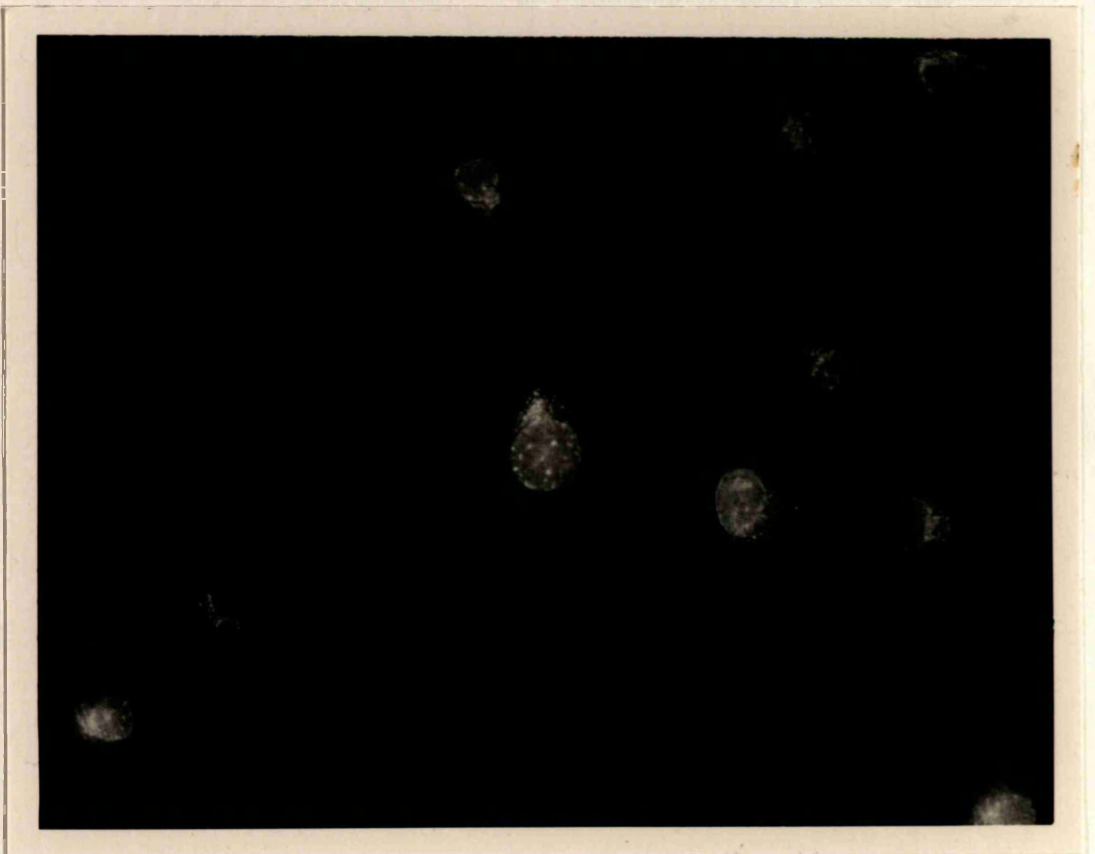
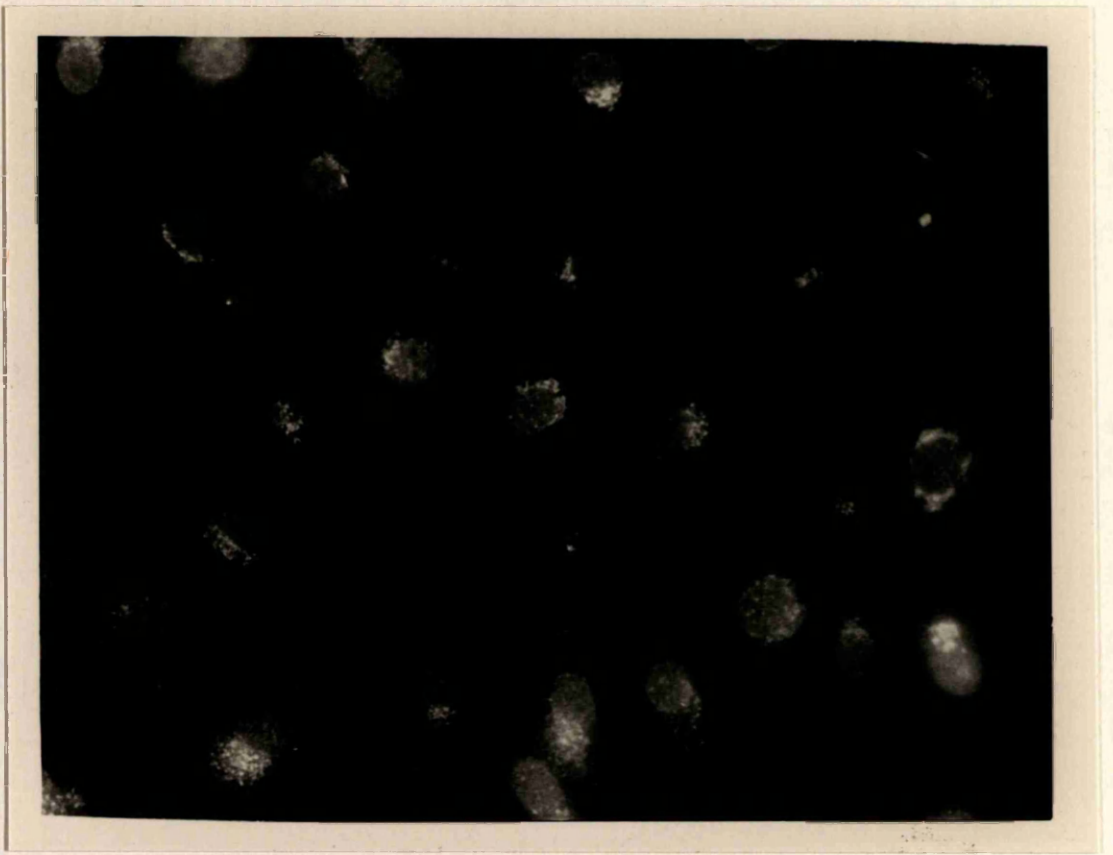
Immunofluorescent labelling of Golgi apparatus of CrFK cells with anti-p58 antibody.

- A. Drug-free.
- B. Drug-treated.

Drug concentration used was 300uM CCX

Total of 300 cells were counted of which in drug treated and untreated 89% showed perinuclear staining.

A



B

FIGURE 16.

Immunofluorescent labelling of Golgi apparatus of MDBK cells with anti-p58 antibody.

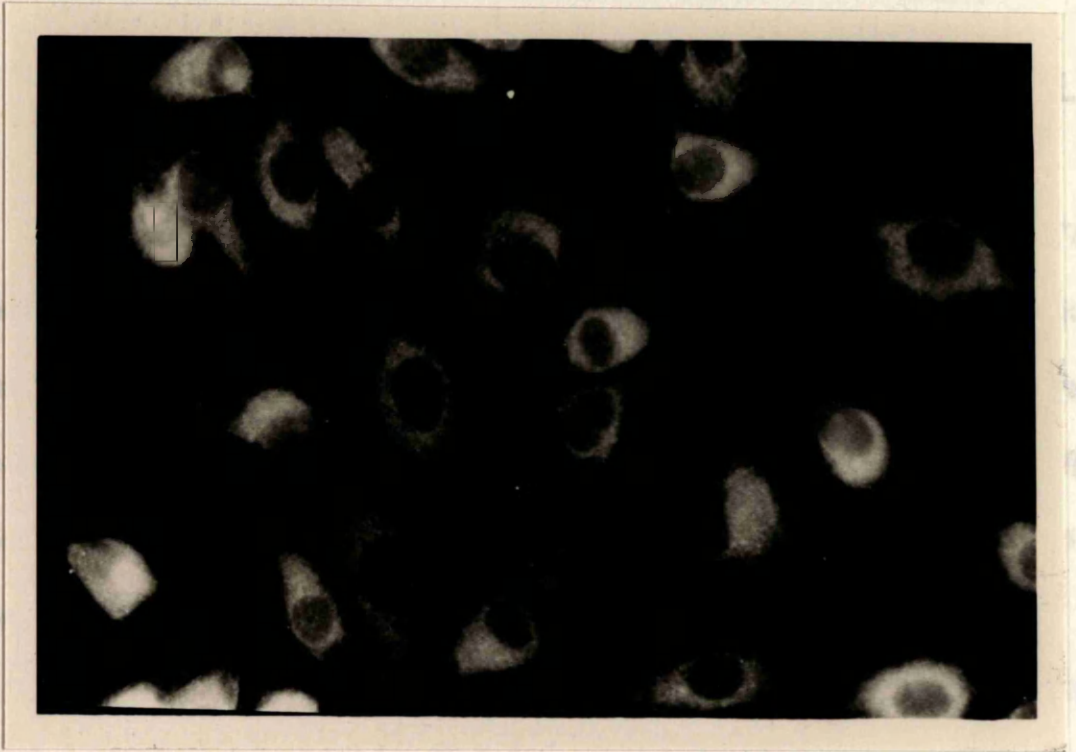
A. Drug-free.

B. Drug-treated.

Drug concentration used was 300uM CCX

Total of 300 cells were counted of which 91% of untreated cells showed perinuclear or cytoplasmic staining, while in drug treated cells only 11% showed perinuclear or cytoplasmic staining.

A



B

3.1.8. INVESTIGATION OF THE EFFECT OF OUABAIN IN COMBINATION WITH CCX, ON THE P58 GOLGI STAINING OF MDBK CELLS.

One of the known effects of triterpenoid treatment of cells is perturbation of Na^+/K^+ ATPase regulation, resulting in decreased intracellular level of Na^+ and concomitant increase in the level of intracellular K^+ . In order to investigate whether the Na^+/K^+ ATPase-induced ion fluxes played a role in the effect of the drug on the Golgi apparatus, the effect of the Na^+/K^+ ATPase inhibitor ouabain on the p58 Golgi protein was investigated in immunofluorescence experiments either by itself or when in combination with CCX.

MDBK cells were seeded at 2×10^5 cells/well on glass coverslips in 24 well Nunc trays. After overnight incubation the cells were maintained in the absence of any drug or overlaid with medium containing, CCX, ouabain, or ouabain and CCX in combination and incubated at 37°C for 5h. The cells were then processed for immunofluorescence with the antibody against the p58 Golgi protein as previously described.

Figures(17A, 17B, and 17C) show representative micrographs obtained from this experiment. In the absence of any drug cytoplasmic and perinuclear(Golgi) staining are evident (17A). Cells treated with CCX alone lacked perinuclear or cytoplasmic (Golgi) staining (17B). The combination of CCX and Ouabain resulted in the detection of cytoplasmic and perinuclear (Golgi) staining (17C).

The data suggest firstly that the modulation of Na^+/K^+ ATPase activity by CCX is involved in the effect of the drug on the Golgi apparatus, and secondly that Ouabain ameliorates the effect of CCX treatment on the Na^+/K^+ ATPase activity.

Ouabain alone had no effect (data not shown).

FIGURE 17.

Immunofluorescent labelling of Golgi apparatus of MDBK cells with anti-p58 antibody.

A-Untreated

B-CCX-treated

C-CCX + Ouabain-treated

Drug concentration used was 300uM CCX

Total of 300 cells were counted of which 71% showed perinuclear staining in the untreated cells. In Ouabain + CCX-treated cells 78% showed perinuclear staining, while in cells treated with CCX only < 1% showed perinuclear staining.

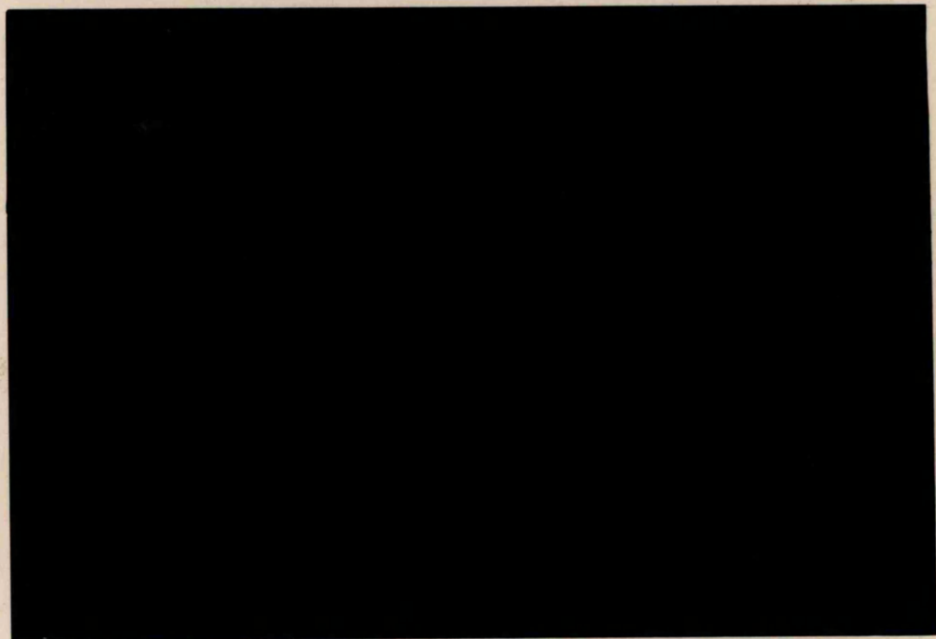
Ouabain alone had no effect on the pattern of perinuclear staining of the cells and resembled the untreated cells (data not shown).

219

A



B



C



3.1.9. DETECTION OF THE ACIDIC COMPARTMENTS OF MDBK CELLS TREATED WITH CCX. USING DAMP/ANTI-DAMP SYSTEM.

Some cell types have acidic trans-Golgi compartments. To investigate whether MDBK cells have an acidic Golgi compartment and to determine the effect of CCX treatment on acidic compartments in these cells, the cells were treated with DAMP [3-92, 4-dinitroanilino 0-3'-amino-N-methyldipropylamine]. DAMP permeates the cells and precipitates at low pH; when the cells are washed the DAMP is removed from the cells except where it has been precipitated in acidic compartments. The acidic compartments, so tagged were detected by monoclonal anti-DAMP and visualised by immunofluorescence.

Figure (18) shows representative micrographs obtained from this experiment. In the absence of CCX perinuclear immunofluorescence was evident, although many discrete spots throughout the cytoplasm were also labelled (18A). The tagged structures illuminated by the FITC-anti DAMP antibody are expected to include Golgi apparatus, trans-Golgi transport vesicles, endosomes, and lysosomes (18A). In CCX treated cell cultures however no perinuclear (Golgi) staining was evident although punctate cytoplasmic staining was seen (Fig 18B).

As the MDBK cells displayed perinuclear fluorescence typical of the Golgi apparatus, it is concluded that MDBK cells have an acidic Golgi compartment. The fluorescence pattern obtained in the presence of CCX can be explained by the suggestion that CCX treatment neutralises the acidic compartments of the Golgi apparatus, or that the acidic compartments of the Golgi apparatus have been dispersed in the form of multiple vesicles and does not exist as in the form of a discrete trans-Golgi stack.

FIGURE 18.

Immunofluorescent labelling of MDBK cells with DAMP, anti-DAMP antibody.

A.untreated

B.CCX-treated

Drug concentration used was 300uM CCX

Total of 300 cells were counted of which 88% of untreated cells showed perinuclear staining while, <1% of drug-treated cells showed perinuclear staining.

A



giving an effect on cell culture viability (Table 1). In the presence of FeLV-A, the replication of FeLV-A in dividing and non-dividing FeA cell cultures.

FeA resting cell cultures were prepared as described in Material and Methods, (Section 2.2.4.). FeA cell monolayers [2×10^5 cells/well (non-resting cells), and 5×10^5



B

3.2. INVESTIGATION OF THE ANTIVIRAL EFFECT OF TRITERPENOID COMPOUNDS ON THE REPLICATION OF FELINE LEUKAEMIA VIRUS TYPE-A (FeLV-A).

3.2.1. ONE STEP GROWTH CURVE OF FeLV-A IN RESTING AND NON-RESTING FeA CELL CULTURES.

As triterpenoid drug treatment can impair the replication of FeA cells [e.g. one day treatment with 150 μ M CCX inhibited FeA cell culture growth by 10% (table 1A)] without having an effect on cell culture viability (table 1A), it was important to investigate the replication of FeLV-A in dividing and non-dividing FeA cell cultures.

FeA resting cell cultures were prepared as described in Material and Methods, (section 2.2.4.). FeA cell monolayers [2×10^5 cells/well (non-resting cells), and 5×10^5 cells/60mm tissue culture plate (resting cells)] were infected with FeLV-A at a m.o.i. of 5 p.f.u./cell. After the absorption period the infected monolayers were washed 3 times with PBS, and incubated at 37°C. At 0, 3, 6, 9, 12, 24, 36, and 48h PI, the culture medium was pipetted-off the infected cells, and the infectivity titrated on QN10S cell monolayers.

Fig 19 shows the one step growth cycle of FeLV-A in resting and non-resting FeA cell cultures. The curve obtained for FeLV-A infected non-resting cell cultures shows that the virus exited the eclipse phase at about 12h PI, replicated exponentially until about 24h PI yielding 0.44 p.f.u./cell, and reached a maximum infectious yield of 3.7 p.f.u./cell at 48h PI. In other FeLV-A one step growth experiments (data not shown) the growth curve reached a plateau at 24h PI (yielding 6.5 p.f.u./cell and reaching 8 p.f.u./cell after 48h PI),

FIGURE. 19

therefore it was concluded that the length of the FeLV-A growth cycle in FeA cell cultures was between 24-48h PI. In resting cell cultures, infectious progeny virus was not detected before 24h PI, but thereafter there was a small increase (0.002 p.f.u./cell) in the infectious virus yield at 36-48h PI.

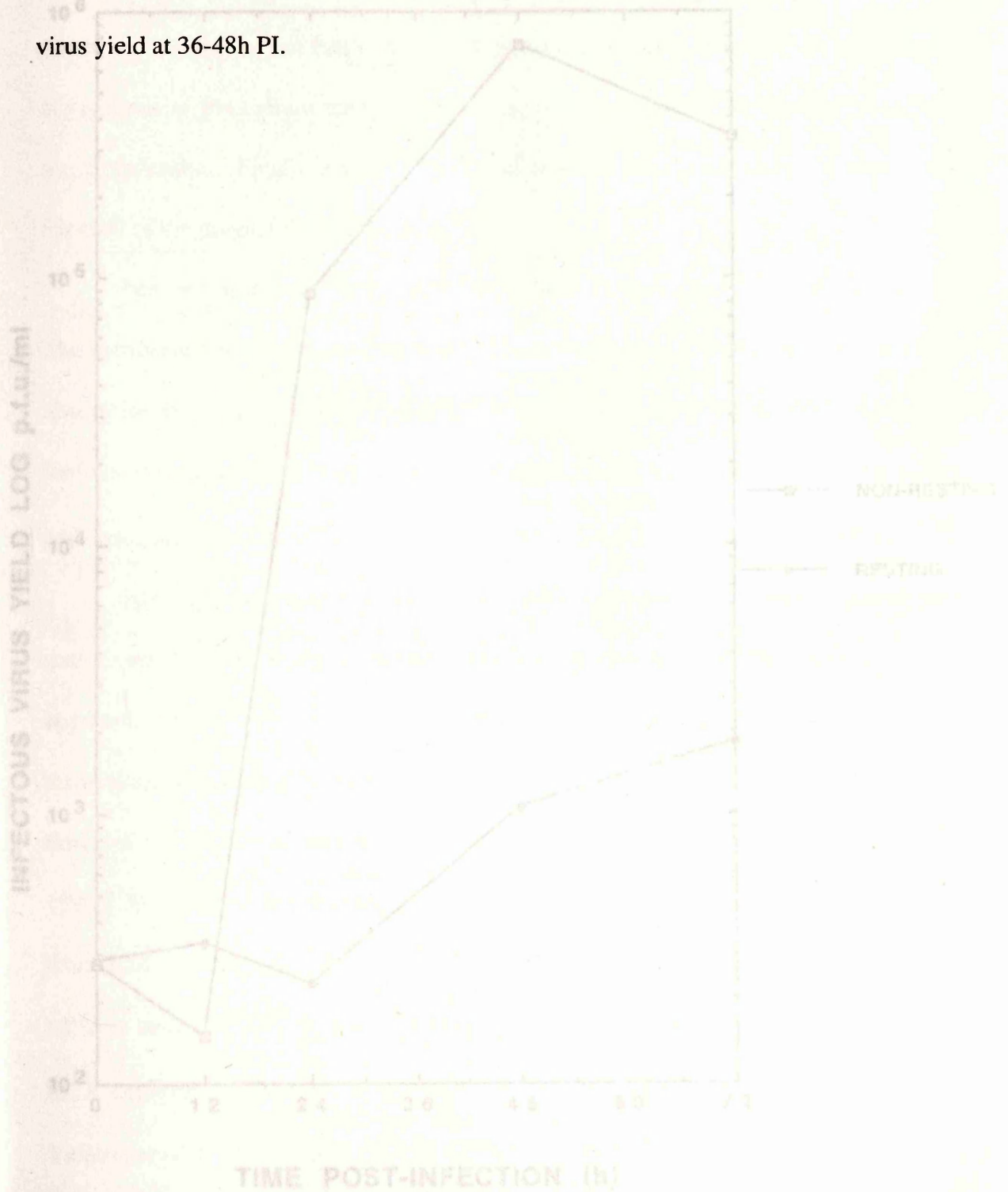
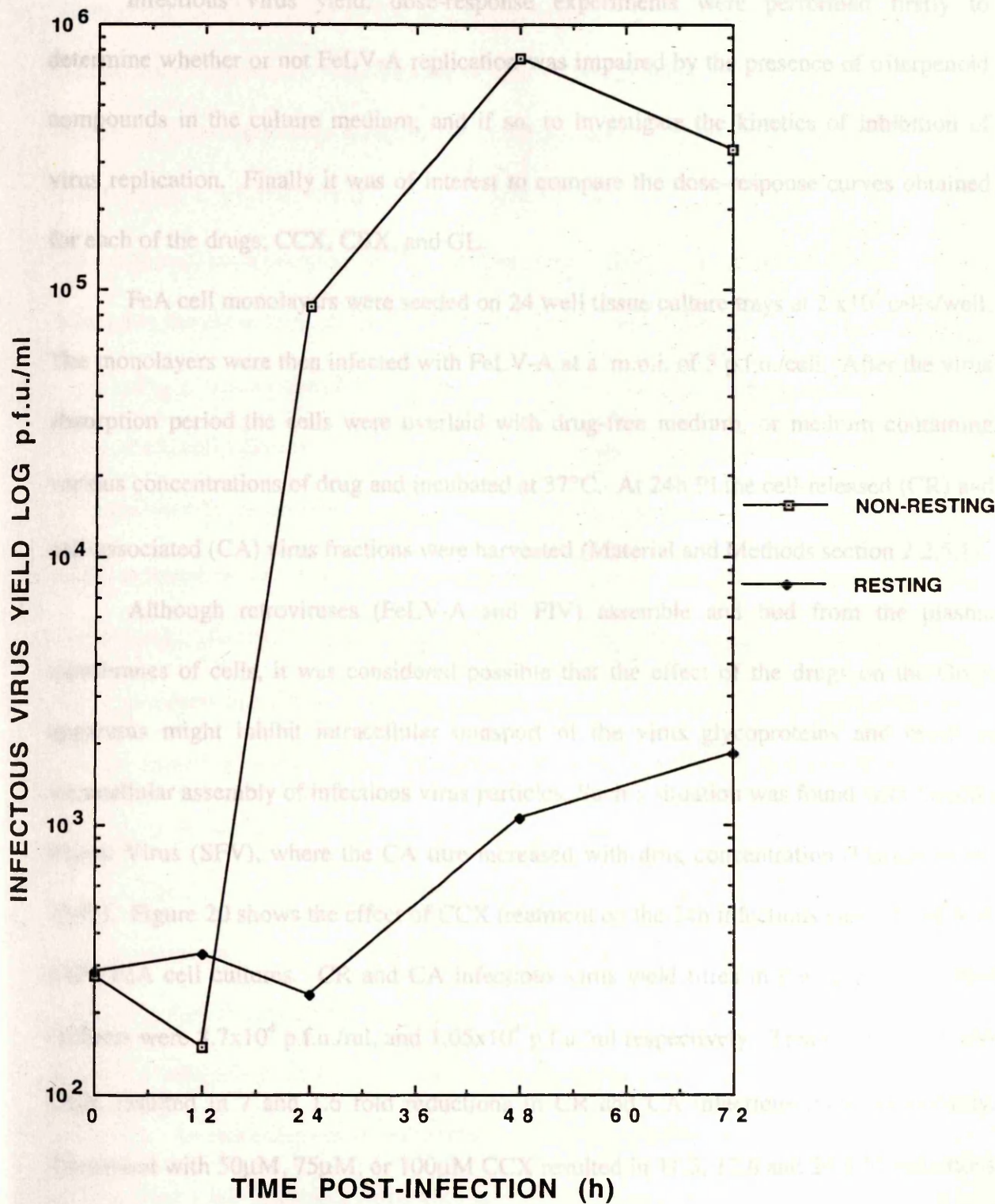


FIGURE. 19
ONE STEP GROWTH OF FeLV-A IN
RESTING AND NON-RESTING FeA
CELL CULTURES.



3.2.2. FeLV-A INFECTIOUS VIRUS YIELD/ CCX: CBX: AND GL

DOSE-RESPONSE EXPERIMENTS.

Infectious virus yield, dose-response experiments were performed firstly to determine whether or not FeLV-A replication was impaired by the presence of triterpenoid compounds in the culture medium, and if so, to investigate the kinetics of inhibition of virus replication. Finally it was of interest to compare the dose-response curves obtained for each of the drugs; CCX, CBX, and GL.

FeA cell monolayers were seeded on 24 well tissue culture trays at 2×10^5 cells/well. The monolayers were then infected with FeLV-A at a m.o.i. of 5 p.f.u./cell. After the virus absorption period the cells were overlaid with drug-free medium, or medium containing various concentrations of drug and incubated at 37°C. At 24h PI the cell-released (CR) and cell-associated (CA) virus fractions were harvested (Material and Methods section 2.2.5.1).

Although retroviruses (FeLV-A and FIV) assemble and bud from the plasma membranes of cells, it was considered possible that the effect of the drugs on the Golgi apparatus might inhibit intracellular transport of the virus glycoproteins and result in intracellular assembly of infectious virus particles. Such a situation was found with Semliki Forest Virus (SFV), where the CA titre increased with drug concentration (Dargan et al., 1992). Figure 20 shows the effect of CCX treatment on the 24h infectious yield of FeLV-A from FeA cell cultures. CR and CA infectious virus yield titres in the drug-free control cultures were 8.7×10^4 p.f.u./ml, and 1.05×10^4 p.f.u./ml respectively. Treatment with 25µM CCX resulted in 7 and 1.6 fold reductions in CR and CA infectious titres respectively. Treatment with 50µM, 75µM, or 100µM CCX resulted in 11.5, 12.6 and 24 fold reductions in CR infectious virus titres respectively. CA infectivity also decreased in a dose-dependent

manner; treatment with 50 μ M, 75 μ M, or 100 μ M CCX resulted in 1.7, 3.7, and 3.5 fold reductions in infectious virus yield respectively. Treatment with 150 μ M CCX resulted in a more pronounced decrease in CR and CA infectious virus yield titres (155, and 191 fold reductions respectively). The data show; that CCX has antiviral activity against FeLV-A, inhibiting virus replication by up to 190 fold; that the CR infectivity diminishes at a faster rate than the CA infectivity (except at 150 μ M CCX); and that the kinetics of the CR curve are tri-phasic, i.e. a pronounced loss of infectivity at low concentration, followed by a near plateau in the curve until about 100 μ M CCX, then a greater decrease with 150 μ M CCX..

Fig 21 shows the effect of CBX treatment on the 24h infectious yield of FeLV-A from FeA cell cultures. The CA and CR infectious virus yields from drug-free control cultures were those given in Fig 20. Treatment of FeLV-A infected cell cultures with 25 μ M CBX resulted in 11, and 2.3 fold reductions in the CR and CA infectious virus yields respectively. Treatment with 50 μ M, 75 μ M, or 100 μ M CBX resulted in 6.6, 14.7, and 17.1 fold reductions in the CR infectivity respectively, and 4.6, 3.5, and 8.1 fold reductions in the CA infectivity respectively. Treatment with 150 μ M CBX resulted in 102 and 12 fold reductions in CR, and CA infectious virus yields respectively.

The data show that CBX treatment of FeLV-A infected FeA cell cultures inhibited virus replication by up to about 100-fold. The kinetics of the CR dose-response curve was similar to that obtained after treatment with CCX. CR infectivity decreased at a faster rate than CA infectivity, and the CR curve displayed the tri-phasic kinetics seen with CCX treatment. In this experiment, uninfected cultures were treated with CBX in parallel; these controls for CBX-induced cytotoxicity revealed that cell culture viability remained > 90%

(for all of the drug concentrations), while replication indices of 1.5, 1.6, 1.4, 1.2, 1.6, and 1.2 were obtained for FeA cell cultures treated with 0 μ M, 25 μ M, 50 μ M, 75 μ M, 100 μ M or 150 μ M CBX respectively. The replication indices suggest that it is unlikely that the more pronounced decrease in infectious yield of FeLV-A observed after treatment with 100 μ M-150 μ M CBX can be explained simply by inhibition of the growth of FeA cell culture.

Figure 22 shows the effect of GL treatment on the 24h infectious virus yield of FeLV-A from FeA cell cultures. The infectious virus yields from the CR and CA fractions for the drug-free control cultures were 1.34×10^5 p.f.u./ml, and 2×10^4 p.f.u./ml respectively. Treatment of the infected FeA cell cultures with 500 μ M GL resulted in 5.6 and 3 fold reductions in CR and CA infectious virus yields respectively. Treatment with 1000 μ M, 1500 μ M, 2000 μ M, or 2500 μ M GL resulted in decreases in the CR infectivity of 5.9, 6.5, 11.1, and 128 fold respectively, while decreases in CA infectivity were 8, 4.7, 7.9, and 33 fold respectively. Uninfected FeA control cell cultures treated in parallel with GL revealed that cell culture viability was > 90% for all drug concentrations, and RI values were 2.5, 1.7, 1.7, 1.4, 1.5, and 1.0 for cultures treated with 0 μ M, 500 μ M, 1000 μ M, 1500 μ M, 2000 μ M, or 2500 μ M GL respectively.

The kinetics of the CR dose-response curve were similar to those obtained with infected FeA cell cultures treated with CCX, or CBX. The CR curve kinetics were triphasic, reaching a plateau when treated with > 500 μ M GL and decreasing again when treated with 1500 μ M GL. Although the decrease in CR infectivity was greater than the CA infectivity decrease when cell cultures were treated with 500 μ M GL, the CA titre reduction

was greater than the CR titre reduction when the cultures were treated with 1000 μ M or 2000 μ M GL.

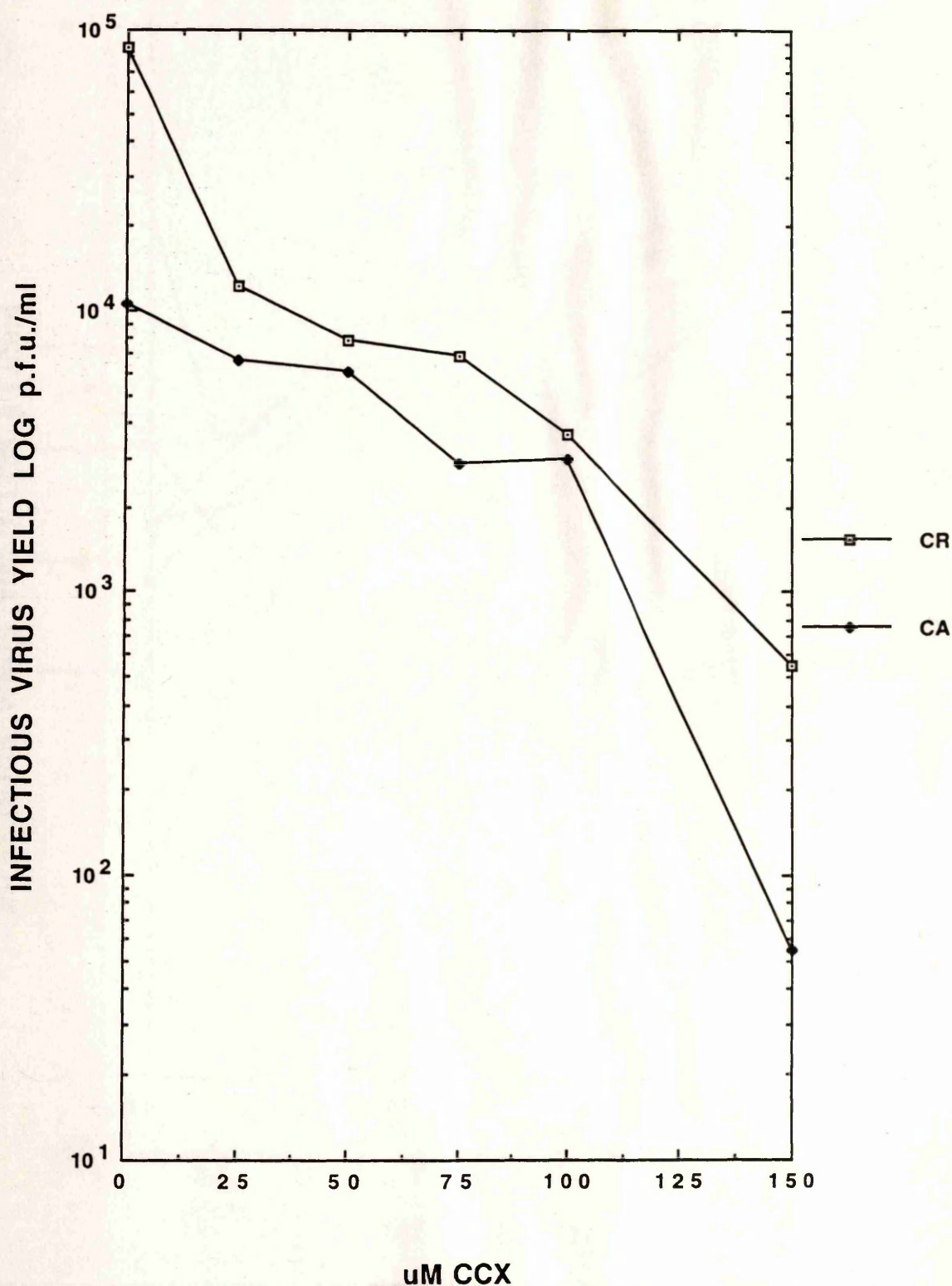
It is concluded, from the data shown in Figures 20, 21, and 22 that each of the triterpenoid compounds has antiviral activity against FeLV-A. Treatment of the infected cell cultures with 150 μ M CCX or CBX resulted in an infectious yield reduction of about 100-150 fold, while treatment with 2500 μ M GL gave 128 fold reduction.

In general, the infectivity in the CR fractions decreased at a faster rate, than the CA infectivity. At least two explanations can be given for this; Firstly the drug might have virucidal activity and so directly inactivate virus particles in the culture medium, secondly the drug might impair release of infectious virus from the infected cell surface.

The kinetics of the CR dose-response curves obtained for each drug were similar; an initial pronounced decrease in the CR titre when cell cultures were treated with low concentrations of drugs, followed by a near plateau in the curve when treated with higher concentrations. Two possible explanations present themselves; Firstly there might exist a low level population of CCX resistant (CCX^r) virus in the virus stock used. Secondly the drug might inhibit a function which enhances, but is not strictly essential for virus growth.

It is possible that impairment of cell culture replication might have contributed to the loss of CR and CA infectivity in the cell cultures treated with 150 μ M CCX or CBX. 150 μ M CCX or CBX were the limiting concentrations determined from earlier cytotoxicity tests, and it is probable that the physiological condition of the cell cultures used in each experiment could affect the precise concentration of the drugs at which cell culture growth is impaired.

FIGURE. 20
The EFFECT OF CCX TREATMENT ON
THE 24H INFECTIOUS YIELD.
OF FeLV-A FROM FeA CELLS.



The effect of CCX, or CBX on the 24h infectious yield of FeLV-A from FeA cells.

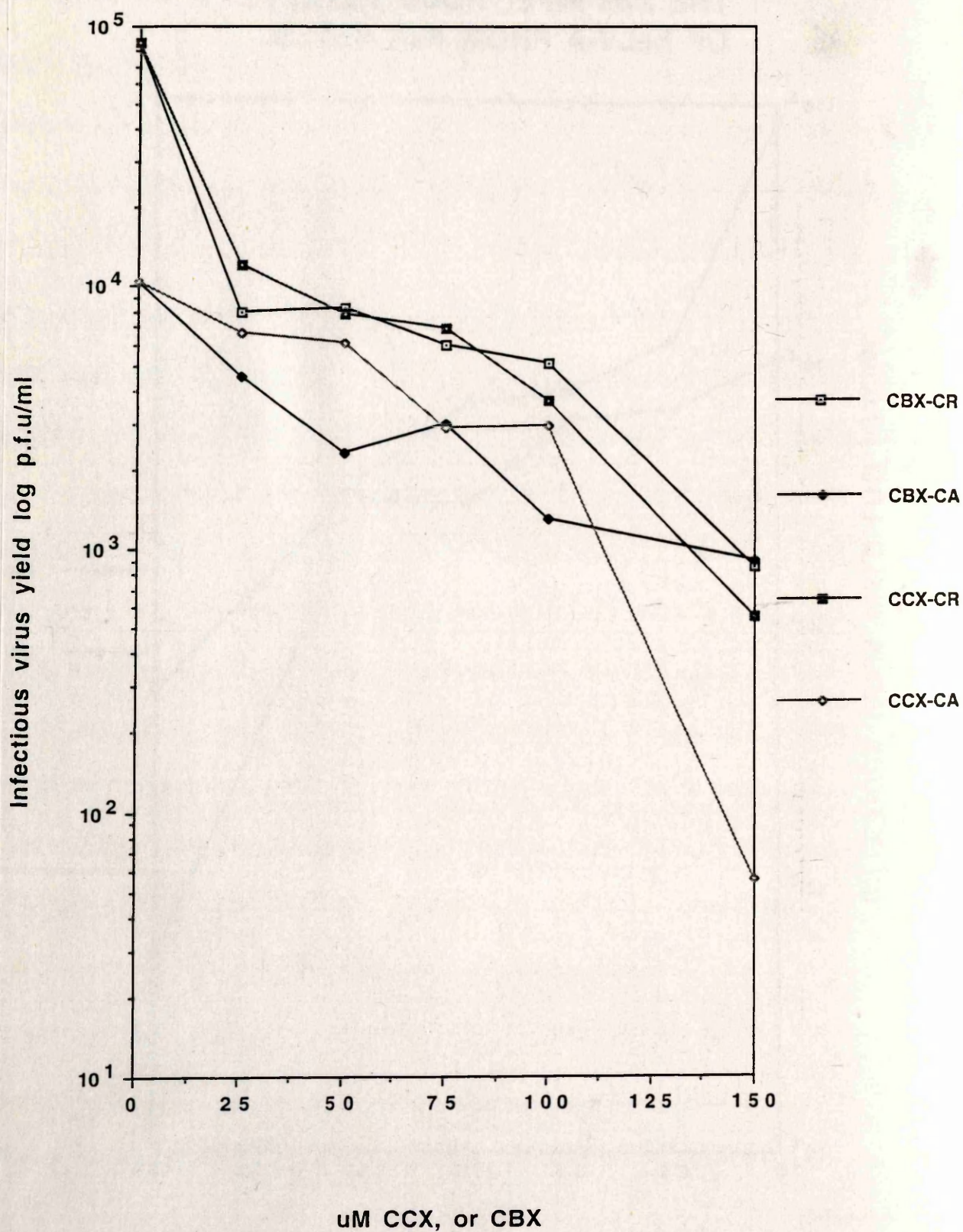
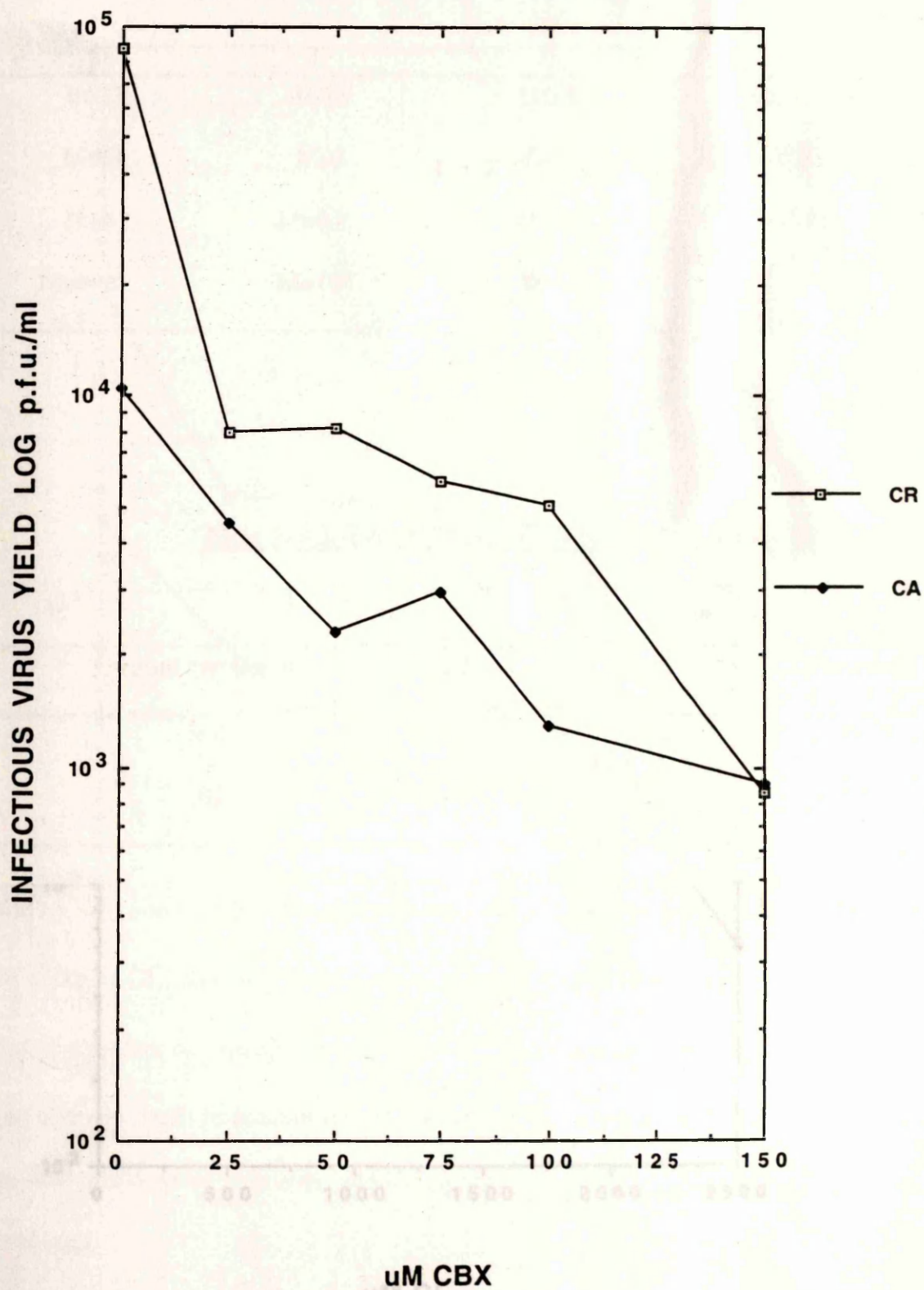


FIGURE. 21
THE EFFECT OF CBX TREATMENT ON
THE 24H INFECTIOUS YIELD.
OF FeLV-A FROM FeA CELLS.



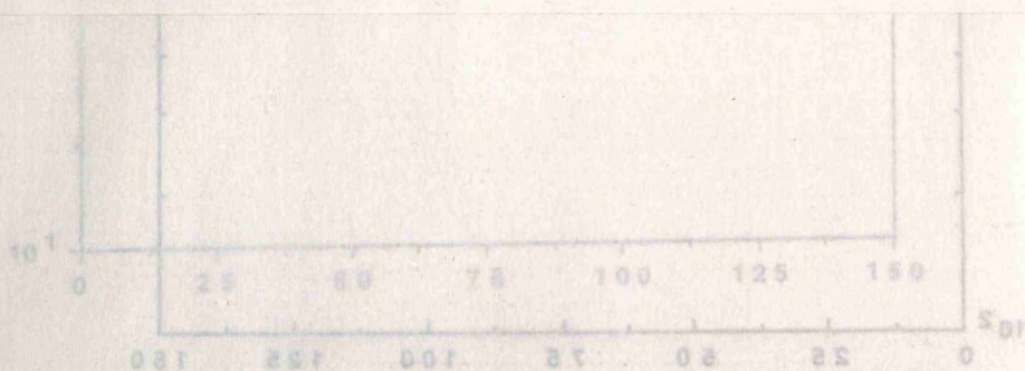
The effect of CCX, or CBX 45 1580017

Concentration of the drug inhibiting FeLV-A cell released yield by 25%, 50%, or 90% (ED25, ED50, ED90)

Compound	ED25	ED50	ED90
CCX	3.9uM	8uM	42uM
CBX	2.8uM	5.6uM	24uM
GL	48uM	191uM	2000uM

Determination of selectivity index of CCX and CBX on FeLV-A yield.

Compound	IC25	ED25	selectivity index
CBX	24uM	2.8uM	8.6
CCX	70uM	3.9uM	18



uM CCX, or CBX
CBX

FIGURE. 22
THE EFFECT OF GL TREATMENT ON
THE 24H INFECTIOUS YIELD.
OF FeLV-A FROM FeA CELLS.

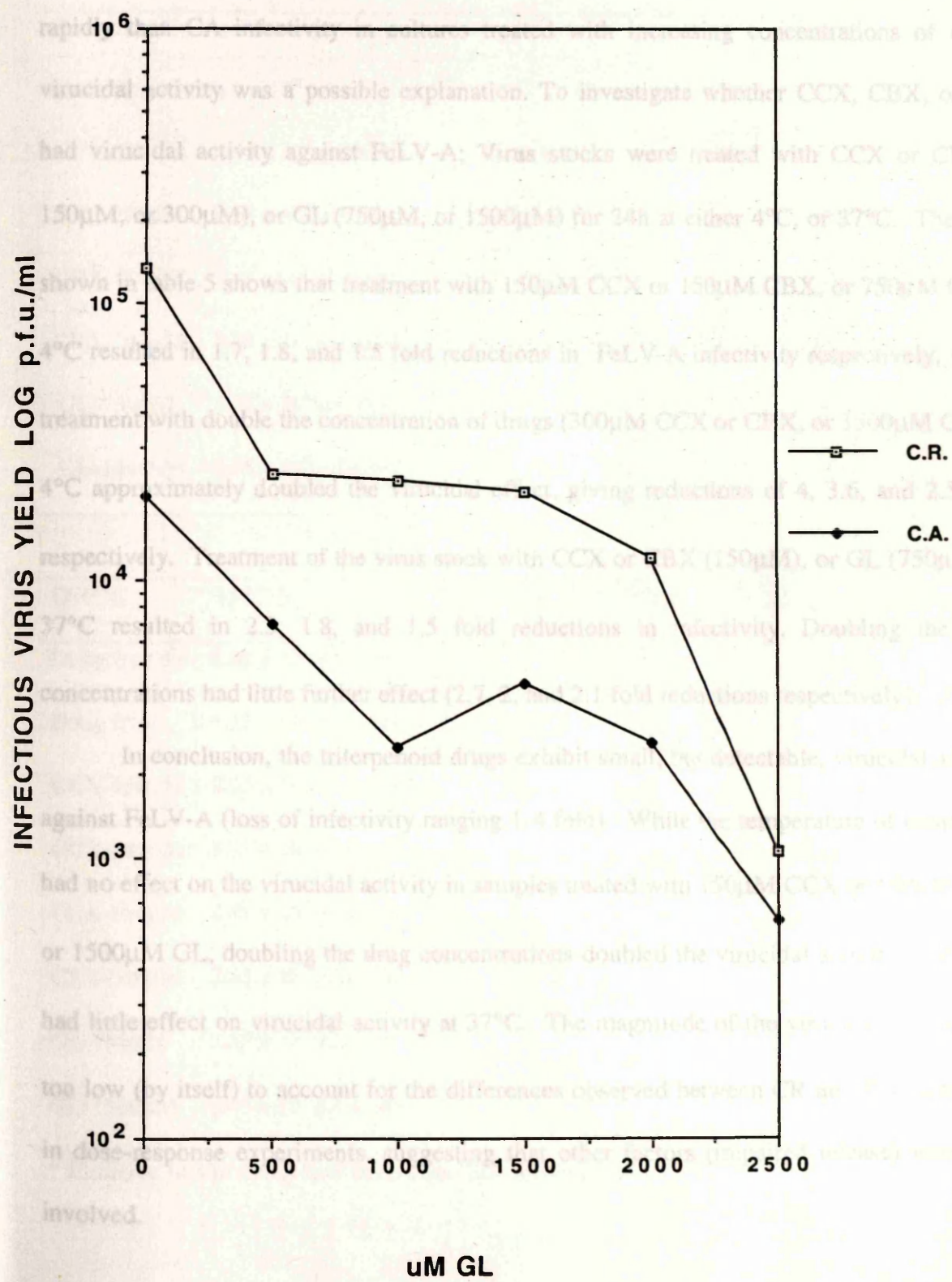


TABLE 5. FeLV-A: VIRUCIDAL ACTIVITY OF CCX, CBX,

3.2.3. ESTIMATION OF THE VIRUCIDAL ACTIVITY OF CCX.

CBX. AND GL AGAINST FeLV-A.

Drug-dose response experiments showed that CR infectivity decreased more rapidly than CA infectivity in cultures treated with increasing concentrations of drug: virucidal activity was a possible explanation. To investigate whether CCX, CBX, or GL had virucidal activity against FeLV-A; Virus stocks were treated with CCX or CBX (150µM, or 300µM), or GL (750µM, or 1500µM) for 24h at either 4°C, or 37°C. The data shown in table 5 shows that treatment with 150µM CCX or 150µM CBX, or 750µM GL at 4°C resulted in 1.7, 1.8, and 1.5 fold reductions in FeLV-A infectivity respectively, while treatment with double the concentration of drugs (300µM CCX or CBX, or 1500µM GL) at 4°C approximately doubled the virucidal effect, giving reductions of 4, 3.6, and 2.5 fold respectively. Treatment of the virus stock with CCX or CBX (150µM), or GL (750µM) at 37°C resulted in 2.3, 1.8, and 1.5 fold reductions in infectivity. Doubling the drug concentrations had little further effect (2.7, 2, and 2.1 fold reductions respectively).

In conclusion, the triterpenoid drugs exhibit small, but detectable, virucidal activity against FeLV-A (loss of infectivity ranging 1-4 fold). While the temperature of incubation had no effect on the virucidal activity in samples treated with 150µM CCX or 150µM CBX or 1500µM GL, doubling the drug concentrations doubled the virucidal activity at 4°C but had little effect on virucidal activity at 37°C. The magnitude of the virucidal activity was too low (by itself) to account for the differences observed between CR and CA infectivity in dose-response experiments, suggesting that other factors (impaired release) were also involved.

TABLE 5. FeLV-A: VIRUCIDAL ACTIVITY OF CCX, CBX, AND GL DETERMINED AFTER 24H INCUBATION AT 4°C (A) OR 37°C (B).

A

DRUG	TITRE	FOLD REDUCTION	% REDUCTION*
Drug free 0h	1.42 x10 ⁷ p.f.u./ml	-	-
Drug free	6.6 x 10 ⁶ p.f.u./ml	-	-
CCX-150µM	3.95 x 10 ⁶ p.f.u./ml	1.7	40.15
CCX-300µM	1.65 x 10 ⁶ p.f.u./ml	4	75
CBX-150µM	3.65 x10 ⁶ p.f.u./ml	1.8	44
CBX-300µM	1.85 x 10 ⁶ p.f.u./ml	3.6	72
GL-750µM	4.35 x 10 ⁶ p.f.u./ml	1.5	34.1
GL-1500µM	2.65 x 10 ⁶ p.f.u./ml	2.5	59.8

B

DRUG	TITRE	FOLD REDUCTION	% REDUCTION*
Drug free 0h	1.42 x 10 ⁷ p.f.u./ml	-	-
Drug free	5.25 x 10 ⁶ p.f.u./ml	-	-
CCX-150µM	2.25 x 10 ⁶ p.f.u./ml	2.3	57.14
CCX-300µM	1.95 x 10 ⁶ p.f.u./ ml	2.7	62.85
CBX-150µM	2.85 x 10 ⁶ p.f.u./ml	1.8	45.71
CBX-300µM	2.65 x 10 ⁶ p.f.u./ml	2.0	49.5
GL-750µM	3.55 x 10 ⁶ p.f.u./ml	1.5	32.8
GL-1500µM	2.45 x 10 ⁶ p.f.u./ml	2.1	53.3

* Relative to the drug-free titre after 24h incubation.

**3.2.4. PURIFICATION OF FeLV-A BY FICOLL DENSITY GRADIENT
CENTRIFUGATION. AND INVESTIGATION OF THE SDS PAGE
POLYPEPTIDE PROFILE OF PURIFIED VIRUS.**

In order to identify FeLV-A protein bands in FeA infected cell protein extracts, it was necessary to obtain an SDS PAGE polypeptide pattern from purified FeLV-A virion proteins. FeA cells were seeded at 2×10^7 cells/roller bottle. After overnight incubation, the cell monolayers were infected in the usual way with 1.4×10^7 p.f.u./roller bottle, and incubated at 37°C . The infected FeA cell cultures were harvested 5 days later as described in Material and Methods (section 2.2.3.1). The infected tissue culture medium was clarified by low speed centrifugation, after which virus particles were pelleted by centrifugation at 12000 r.p.m., at 4°C , for 3h, in a Sorvall GSA rotor. The resuspended virus pellet was layered on top of a 5-15% Ficoll gradient, which was then centrifuged to obtain a purified virus preparation (Material and Methods section 2.2.3.4.). A representative example of the density gradient banded virus preparation is shown in Figure 23. The material from the three visible bands were removed from the tube by side puncture and then pelleted (Material and Methods section 2.2.3.4.). The banded materials were examined by electron microscopy. The top band was composed of cellular membrane debris; the middle band contained spherical FeLV-A particles (Fig 24) at $2.9 \times 10^9/\text{ml}$ and a tailed form of FeLV-A particles at $5.32 \times 10^{11}/\text{ml}$. The bottom band contained only the tailed form of FeLV-A particles at $1.11 \times 10^{11}/\text{ml}$ (Fig 25). The infectivity associated with each band was as follows; top band 4×10^4 p.f.u./ml, middle band 5×10^6 p.f.u./ml, and bottom band 2.2×10^6 p.f.u./ml. Particle/p.f.u. ratios calculated for the virus bands Middle $1.05 \times 10^5/1$; and Bottom $5.05 \times 10^5/1$ reveal that most of the virus particles were non-infectious.

FIGURE 23.

FeLV-A particles purified on a 5-15% Ficoll gradient. (Bands 1, 2, and 3 are identified by arrows).

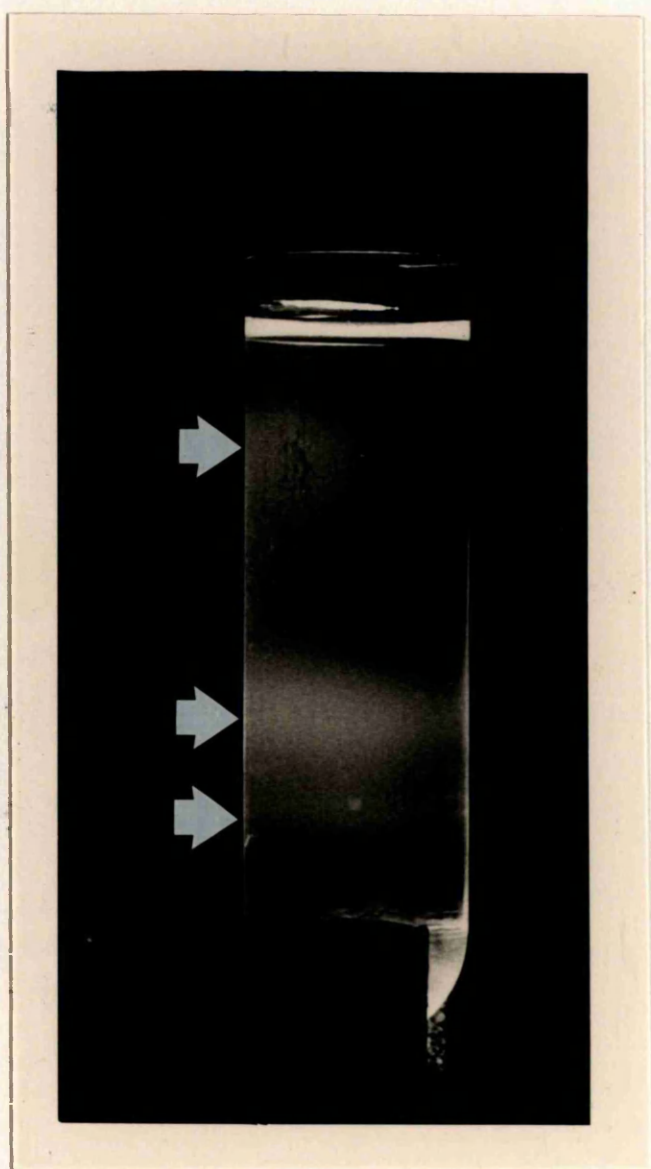


FIGURE 24.

Electron micrograph of purified Ficoll-banded FeLV-A virus, middle band.

Scale bar 100nm.



FIGURE 25.

Electron micrograph of purified Ficoll-banded FeLV-A virus, bottom band.

Scale bar 100nm.



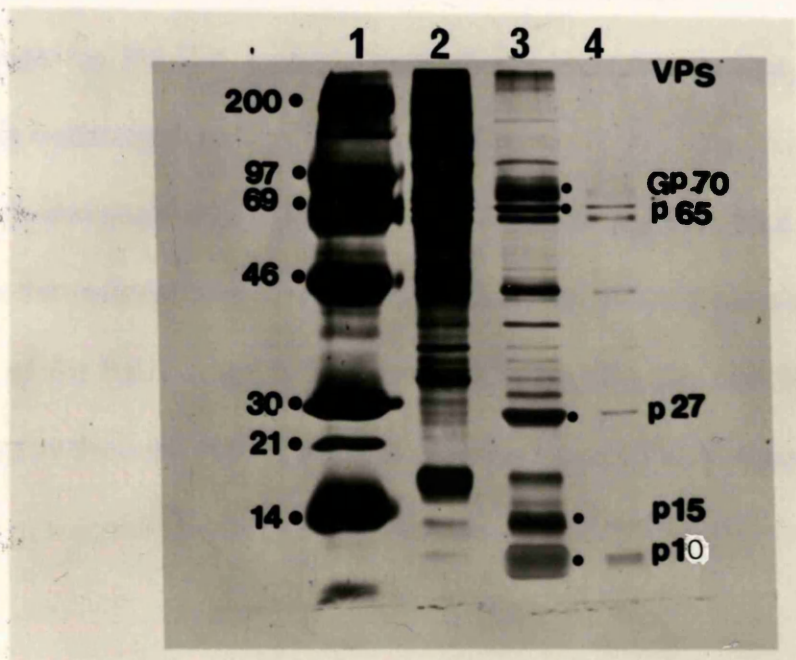
FIGURE 26.

Silver-stained polypeptide profiles of purified FeLV-A particles obtained on a 10% SDS PAGE minigel.

Virus particles were banded on Ficoll gradients.

Molecular weight marker (M) (lane 1), top band (lane 2), middle band (lane 3) and bottom band (lane 4)

- Identified viral proteins (VPs).



3.2.5. The banded materials were solubilized with SDS PAGE extraction buffer and electrophoresed on a 10% SDS PAGE minigel. The resulting gel was silver-stained and is shown in Figure 26. The material in the top band contained mostly cellular proteins, although some FeLV-A proteins (particularly gp70) were present in small amounts. The material in the middle band contained the FeLV-A virion proteins gp70, p65, p27, p15, and p10, but was also contaminated with cellular proteins (as determined by comparison with the polypeptide profile of the top band). The bottom band seemed to contain less protein overall, although the FeLV-A proteins were clearly identified against a background of cellular protein contamination.

Western-immunoblotting (Material and Methods section 2.2.6.3.7) of FeLV-A proteins from the material in the middle band using an infected cat sera, confirmed the identification of the FeLV-A gp70, p27, and p15 bands (Fig 27, lane 8). The antiserum failed to recognise the band of 65K which is probably the p65 FeLV-A reverse transcriptase protein (RT).

It was then investigated: FeLV-A cell cultures were infected with FeLV-A at m.o.i. of 5 p.f.u./cell, incubated at 37°C for 24h or 48h, the infected cell extracts electrophoresed on SDS PAGE gels, and the FeLV-A proteins identified by immunoblotting using the FeLV-A-infected cat serum. As before no FeLV-A proteins were detected. The experiment was again repeated for FeLV-A infection of cells immunoprecipitated, electrophoresed on SDS PAGE gels, and then immunoblotted using the infected cat serum, but again no FeLV-A proteins were detected. It was concluded that infection of the cell cultures with FeLV-A at a m.o.i. of 5 p.f.u./cell followed by incubation for 48h either resulted in a poor infection, or a poor detection.

3.2.5. SDS PAGE POLYPEPTIDE ANALYSIS OF FeLV-A INFECTED FeA CELL

EXTRACTS

In order to investigate the inhibition of FeLV-A replication in the presence of triterpenoid drugs, it was necessary to study the synthesis and processing of FeLV-A proteins in infected cells. This proved a difficult task.

Initial attempts were made to identify FeLV-A proteins in infected cell extracts, by infecting the cell cultures at a m.o.i. of 3-5 p.f.u./cell, labelling with ^{35}S methionine for 24h or 48h, followed by SDS PAGE analysis. However FeLV-A proteins could not be identified in the autoradiographs. Another attempt was made using the same m.o.i. and radiolabelling conditions; except that the infected cell proteins were extracted with immunoprecipitation extraction buffer, immunoprecipitated using an FeLV-A-infected cat serum, and analysed by SDS PAGE. However, again no virus proteins were detected, even after prolonged exposure of the gels. An alternative approach using western-immunoblotting was then investigated; FeA cell cultures were infected with FeLV-A at m.o.i. of 5 p.f.u./cell, incubated at 37°C for 24h or 48h, the infected cell extracts electrophoresed on SDS PAGE gels, and the FeLV-A proteins labelled by western-immunoblotting using the FeLV-A-infected cat serum. As before, no FeLV-A proteins were detected. The experiment was again repeated but FeLV-A proteins were first immunoprecipitated, electrophoresed on SDS PAGE gel, and then western-immunoblotted using the infected cat serum, but again no FeLV-A proteins were detected. It was concluded that infection of the cell cultures with FeLV-A at a m.o.i. of 5 p.f.u./cell followed by incubation for 48h either resulted in a poor infection, or yielded insufficient

viral proteins for analysis. Consequently, we were forced to use persistently infected cell cultures in order to obtain infected cell polypeptide profiles on SDS PAGE gels.

Figure 27 shows western-immunoblotting of the persistently infected or MI FeA cell cultures. Persistently infected cell cultures were pre-treated with the drug (as appropriate), washed 3 times, and overlaid with growth medium containing either no drug or 50 μ M, or 100 μ M CCX. The western blot showed that there was no significant difference in the quantities, or the apparent molecular weights of FeLV-A proteins (gp70, p27, or p15) produced in FeA cell cultures treated with increasing concentrations of drug. As expected, the anti-FeLV-A antibody failed to tag any protein band in extracts of MI cell cultures.

It is concluded from Fig 27 that the drug had either, no significant effect on FeLV-A protein synthesis, or more probably, that the pool of pre-formed FeLV-A proteins in persistently infected cell cultures masked any effect of the drug.

FIGURE 27.

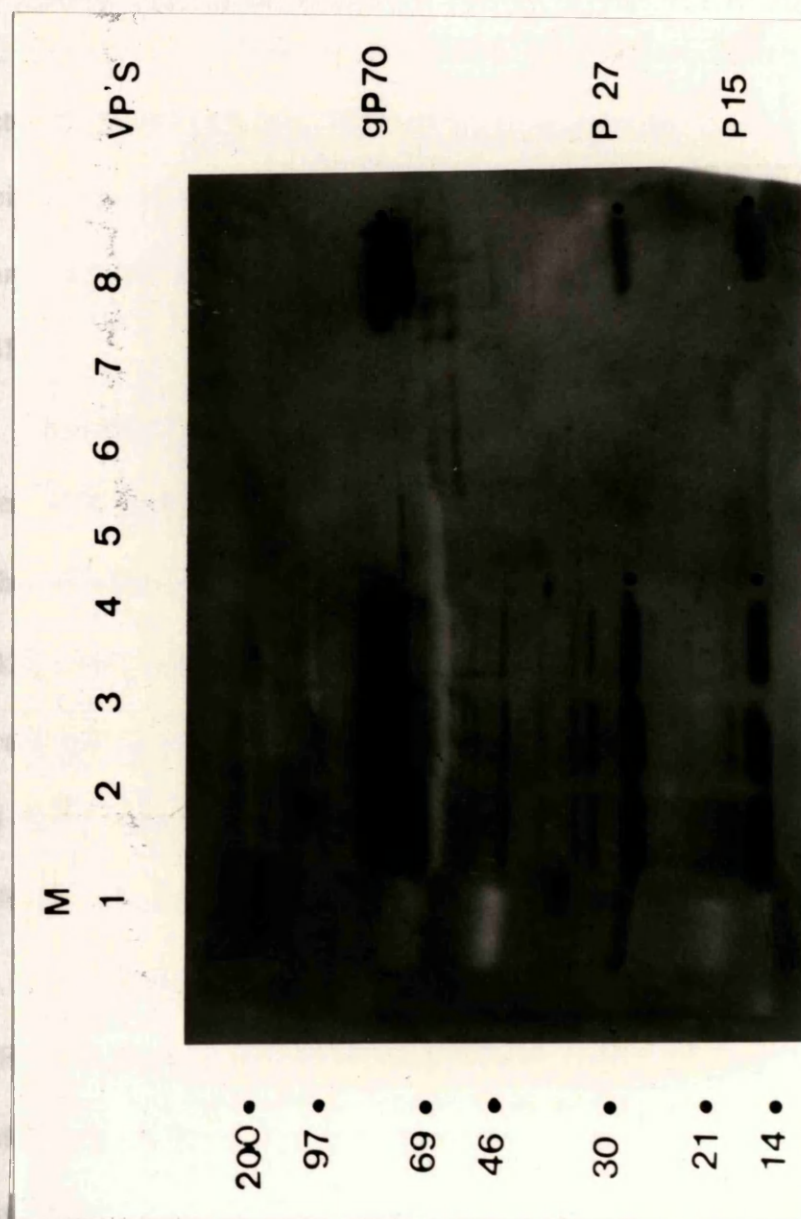
Western blot of the polypeptide profiles obtained on a 10% SDS PAGE gel of FeLV-A infected (lanes 2, 3, and 4) and MI (lanes 5, 6, and 7) FeA cells.

Virus bands were identified using FeLV-A infected cat serum.

Cells were either drug-free (lanes 2 and 5), or treated with 50, or 100 μ M CCX (lanes 3 and 6; 4 and 7 respectively). The polypeptide profiles of purified FeLV-A is shown in lane 8, and the molecular weight markers in lane 1.

- Identified viral proteins (VPs).

3.2.5.1. SDS PAGE ANALYSIS OF RADIO LABELLED PROTEINS IN PROGENY VIRUS PARTICLES, AND IN THE CELL EXTRACTS OF FeLV-A PERSISTENTLY INFECTED CELL CULTURES TREATED WITH CCX.



detected (Fig 29A). In the western blot profile of the purified FeLV-A (Fig 29B), the gp70 band slightly decreased with increasing concentrations of drug, while the p15 band appeared to be unaffected by drug treatment. The P27 band barely detectable in the drug-

3.2.5.1. SDS PAGE ANALYSIS OF RADIOLABELLED PROTEINS IN PROGENY

VIRUS PARTICLES, AND IN THE CELL EXTRACTS OF FeLV-A

PERSISTENTLY INFECTED CELL CULTURES TREATED WITH CCX.

In order to investigate the effect of CCX on nascent protein synthesis in persistently infected FeA cell cultures, ^{35}S -methionine/cysteine was used to label the infected cell proteins. The virus particles, made in the presence of the drug and extruded to the tissue culture medium, were pelleted and their polypeptide composition investigated by SDS PAGE.

Figure 28A, shows the polypeptide profile of mock-infected (MI) FeA cell cultures, treated with increasing concentrations of CCX. Each gel track was loaded with an equal number of c.p.m. Treatment with 100 μM CCX resulted in a decrease in the intensities of the 42K, 30K, and 21K cellular bands. Figure 28B, shows the polypeptide profile of the infected cell extracts, treated with increasing concentrations of CCX. The only FeLV-A band which could be unambiguously identified was gp70, and this appeared to be largely unaffected, when treated with up to 125 μM CCX.

Figure 29, shows the polypeptide profile of the material pelleted from the MI and the FeLV-A persistently infected cell cultures. Due to the low level of radioactivity in the pelleted samples these were investigated by western-immunoblotting using the infected cat serum. Little, or no, labelling of the material pelleted from the MI culture supernatant was detected (Fig 29A). In the western blot profile of the purified FeLV-A virus control, gp70, P27, and p15 were identified by the FeLV-A antiserum (Fig 29B). The intensity of the gp70 band slightly decreased with increasing concentrations of drug, while the p15 band appeared to be unaffected by drug treatment. The P27 band barely detectable in the drug-

free control, was clearly present in the purified virus polypeptide profile, and in the extracts from virus made in the presence of 25 μ M CCX.

The amount of gp70 in infected cell extracts was virtually unaffected by CCX concentrations up to 125 μ M (Fig 28B), but the amount of gp70 in the pelleted virus particles was decreased at 100 μ M CCX (Fig 29 B), suggesting that less gp70 proteins was incorporated into the progeny virus particles. The alternative explanation that fewer virus particles were present at the higher drug concentration samples seems unlikely, given that equal number of c.p.m. were loaded in each gel tract.

FIGURE 28.

Polypeptide profile obtained on a 10% SDS PAGE gel of ^{35}S -methionine/cysteine labelled proteins in the extracts from MI (A), or FeLV-A infected (B) FeA cells.

Cells were either drug-free (lane 1, Figures A and B), or treated with 25, 50, 100, or 125 μM CCX (lanes 2, 3, 4, 5, and 6 respectively, Figures A and B).

The location of the molecular weight markers (M) (non-radiolabelled) resolved on the same gel, have been transferred from the dried gels.

- ▲ Identified viral proteins.
- Bands of unknown origin.

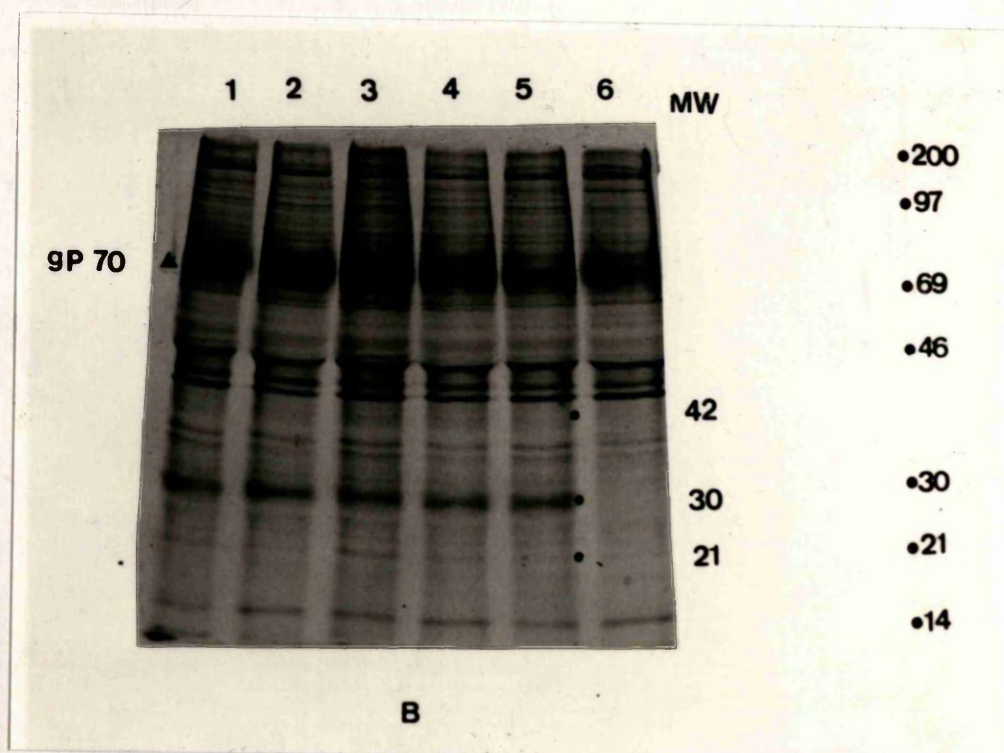
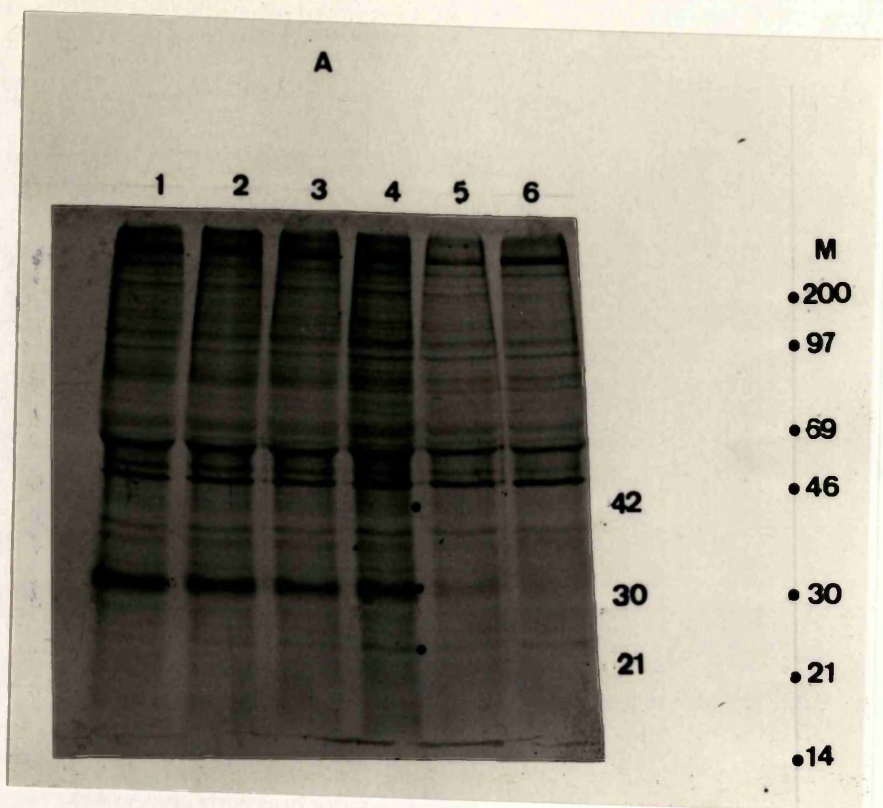


FIGURE 29.

Polypeptide profiles obtained on a 10% SDS PAGE gel of ^{35}S -methionine/cysteine labelled proteins pelleted from the culture medium of MI (A), or FeLV-A infected (B) Fe A cells.

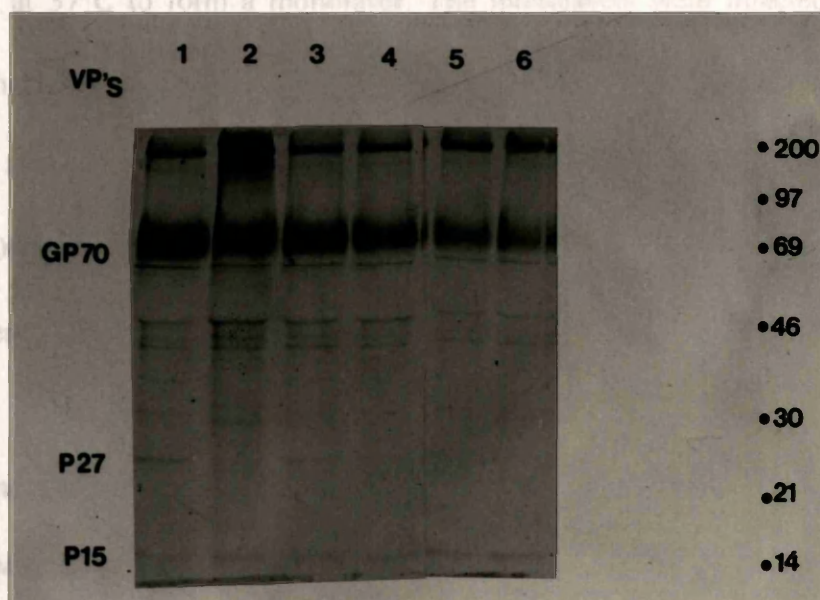
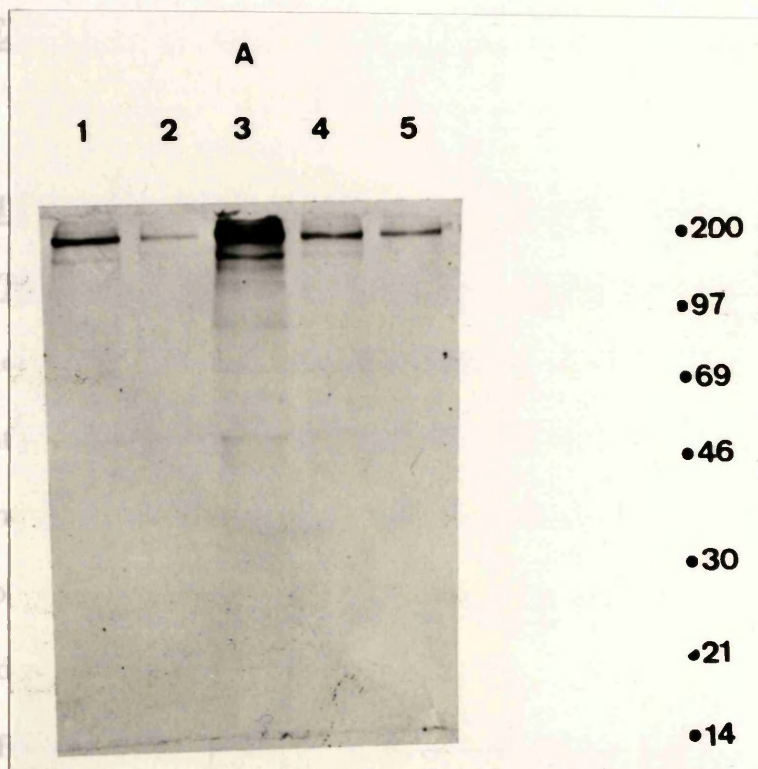
Virus bands were identified by western immunoblotting using FeLV-A infected cat serum. Cells were either drug-free or treated with 25, 50, 100, 125 μM CCX (lanes 1, 2, 3, 4, 5 figure A and lanes 2, 3, 4, 5, and 6 Figure B respectively).

The polypeptide profile of purified FeLV-A is shown in lane 1 of Figure B.

The location of molecular weight markers (M) (non-radiolabelled) resolved on the same gel have been transferred from the dried gels.

Identified viral proteins (VPs).

INVESTIGATION OF THE ANTI-VIRAL EFFECT OF TRITERPENOID COMPOUNDS ON THE REPLICATION OF



3.3. INVESTIGATION OF THE ANTIVIRAL EFFECT OF TRITERPENOID COMPOUNDS ON THE REPLICATION OF FELINE IMMUNODEFICIENCY VIRUS (FIV).

3.3.1. THE EFFECT OF CCX AND CBX TREATMENTS ON THE PLAQUING EFFICIENCY OF FIV ON CrFK CELL MONOLAYERS.

Due to the low titres generally obtained for FIV stocks, dose-response curve experiments of the type used to investigate FeLV-A could not be performed. To investigate whether the triterpenoid compounds had antiviral activity against FIV, and to obtain a measure of antiviral potency, the CCX and CBX ED50 concentrations for FIV were determined.

CrFK cells were seeded on 24 well Nunc trays at 1×10^5 cells/well and incubated overnight at 37°C to form a monolayer. The monolayers were infected with FIV at 100 p.f.u./monolayer. After the virus absorption period, unbound virus was removed by washing, and the cells overlaid with DMEgM 10%FC containing 0µM, 25µM, 50µM, 75µM, 100µM, or 150µM CCX or CBX. The monolayers were incubated at 37°C for 4 days, after which the cell cultures were fixed and virus plaques identified by immunostaining.

The results are shown in Fig30. Treatment with CCX or CBX resulted in a progressive drug-dose dependent reduction in the number of virus plaques produced. The ED50 concentrations obtained were 75µM, and 101µM for CCX and CBX respectively. Thus CCX was more potent than CBX in inhibiting FIV plaques. The tri-phasic kinetics of

the plaquing efficiency curves (particularly that of CBX curve) are reminiscent of the tri-phasic kinetics of the CCX, CBX, and GL dose-response curves obtained for FeLV-A (Figures; 14, 15, and 16 respectively). Treatment with 25 μ M CCX or CBX resulted in an initial decrease in plaquing efficiency of 27%, and 31% respectively. At intermediate concentrations of CCX or CBX the rate of decrease in plaquing efficiency was slowed until cultures were treated with 150 μ M CCX or CBX, when there was a large decrease in plaque number. It is concluded that CCX and CBX have antiviral activity against FIV, and that CCX is slightly more potent than CBX.



Concentration of the drug inhibiting FIV yield by 25 %, 50 %, or 90 % (ED25, ED50, ED90)

Compound	ED25	ED50	ED90
CBX	21uM	101uM	N.D.
CCX	24uM	75uM	121uM

N.D. Not Determined

Determination of selectivity index of CCX and CBX on FIV yield.

Compound	IC25	ED25	selectivity index
CCX	140uM	24uM	5.8
CBX	N.D.	21uM	N.D.

Figure. 30
THE EFFECT OF CCX, AND CBX ON
PLAQUING EFFICIENCY OF FIV.

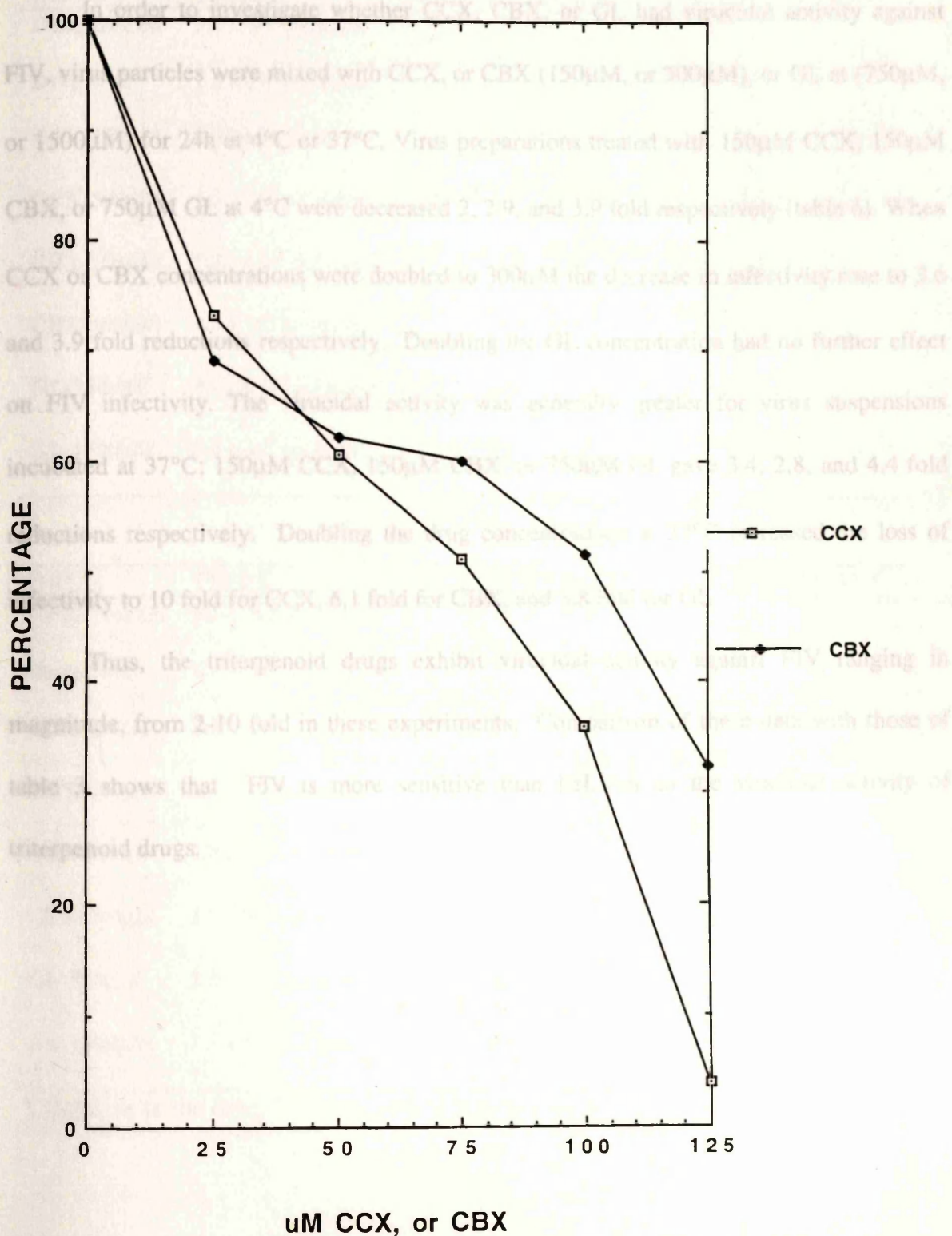


TABLE 6. FIV: VIRUCIDAL ACTIVITY OF CCX, CBX, AND GL DETERMINED AFTER 24H INCUBATION AT 4°C (A)

3.3.2. ESTIMATION OF THE VIRUCIDAL ACTIVITY OF CCX, CBX, OR GL AGAINST FIV.

In order to investigate whether CCX, CBX, or GL had virucidal activity against FIV, virus particles were mixed with CCX, or CBX (150µM, or 300µM), or GL at (750µM, or 1500µM) for 24h at 4°C or 37°C. Virus preparations treated with 150µM CCX, 150µM CBX, or 750µM GL at 4°C were decreased 2, 2.9, and 3.9 fold respectively (table 6). When CCX or CBX concentrations were doubled to 300µM the decrease in infectivity rose to 3.6 and 3.9 fold reductions respectively. Doubling the GL concentration had no further effect on FIV infectivity. The virucidal activity was generally greater for virus suspensions incubated at 37°C; 150µM CCX, 150µM CBX, or 750µM GL gave 3.4, 2.8, and 4.4 fold reductions respectively. Doubling the drug concentrations at 37°C increased the loss of infectivity to 10 fold for CCX, 6.1 fold for CBX, and 5.8 fold for GL.

Thus, the triterpenoid drugs exhibit virucidal activity against FIV ranging in magnitude, from 2-10 fold in these experiments. Comparison of these data with those of table 3 shows that FIV is more sensitive than FeLV-A to the virucidal activity of triterpenoid drugs.

CBX-300µM	3.9×10^7 p.f.u./ml
GL-750µM	2.5×10^2 p.f.u./ml
GL-1500µM	1.9×10^2 p.f.u./ml

* Relative to the drug-free drug after 24h incubation

TABLE 6. FIV: VIRUCIDAL ACTIVITY OF CCX, CBX, AND GL DETERMINED AFTER 24H INCUBATION AT 4°C (A), or 37°C (B)

A

DRUG	TITRE	FOLD REDUCTION	% REDUCTION*
Drug free 0h	9.5 x 10 ³ p.f.u./ml	—	—
Drug free	1.6 x 10 ³ p.f.u./ml	—	—
CCX-150µM	7.92 x 10 ² p.f.u./ml	2	50.5
CCX-300µM	4.5 x 10 ² p.f.u./ml	3.6	71.9
CBX-150µM	5.42 x 10 ² p.f.u./ml	2.9	66.1
CBX-300µM	4.1 x 10 ² p.f.u./ml	3.9	74.4
GL-750µM	4.12 x 10 ² p.f.u./ml	3.9	74.25
GL-1500µM	6.4 x 10 ² p.f.u./ml	2.5	60

B

DRUG	TITRE	FOLD REDUCTION	% REDUCTION*
Drug free 0h	9.5 x 10 ³ p.f.u./ml	—	—
Drug free	1.1 X 10 ³ p.f.u./ml	—	—
CCX-150µM	3.2 x 10 ² p.f.u./ml	3.4	71
CCX-300µM	1.1 x 10 ² p.f.u./ml	10	90
CBX-150µM	1.8 x 10 ² p.f.u. /ml	2.8	64.6
CBX-300µM	3.9 x 10 ² p.f.u./ml	6.1	83.6
GL-750µM	2.5 x 10 ² p.f.u./ml	4.4	77.3
GL-1500µM	1.9 x 10 ² p.f.u./ml	5.8	82.73

* Relative to the drug-free titre after 24h incubation.

3.3.3. PURIFICATION OF FIV BY FICOLL GRADIENT CENTRIFUGATION.

AND INVESTIGATION OF THE SDS PAGE POLYPEPTIDE PROFILE

OF PURIFIED VIRUS.

In order to identify FIV proteins in infected cell extracts, it was first necessary to obtain an SDS PAGE polypeptide profile for the purified virus particles. CrFK cells were seeded overnight at 2×10^7 cells/roller bottle, and the resulting monolayers then infected with FIV at a m.o.i. of 0.001 p.f.u./cell. The infected CrFK cell cultures were harvested 5 days later; The tissue culture medium from the infected culture was clarified by low speed centrifugation, after which the virus particles were pelleted (12000 r.p.m./4°C/3h in a Sorvall GSA rotor). The resuspended virus pellet was layered on top of 5-15% Ficoll gradients, which were then centrifuged (12000 r.p.m./4°C/3h in the Sorvall TST 41 rotor) to band the purified virus. The banded virus preparation is shown in Fig 31. Two broad bands running close together were just visible. After extraction of the banded material, by side-puncture of the tube, the material constituting the bands was examined by electron microscopy. The top band contained spherical FIV particles at 1.1×10^{11} per ml (Figure 32 A), and the bottom band contained the same type of particles at 2.75×10^{11} per ml (Figure 32 B). The infectivity associated with each band was as follows; 3.25×10^3 p.f.u./ml (top band), and 2.8×10^3 p.f.u./ml (bottom band). The particle/p.f.u. calculated for each band were; $3.4 \times 10^7/1$ (top band), and $9.8 \times 10^7/1$ (bottom band), indicating that most of the viruses produced were non-infectious. The banded virus particles were solubilized with SDS PAGE extraction buffer, and the protein extract electrophoresed on a 10% SDS PAGE gel. The resulting gel was silver- stained and is shown in Fig 33A. The polypeptide profile obtained from the top and the bottom bands were virtually identical except that the bottom

band seemed to contain more protein. Comparison of the silver-stained polypeptide profile with a western-blot of the same material using an FIV infected cat serum as the probe, allowed the identification of the FIV gp120, gp41, p24, and p17 proteins. Two further proteins tentatively identified as the FIV p55, and p10 were also resolved in the silver-stained gel.

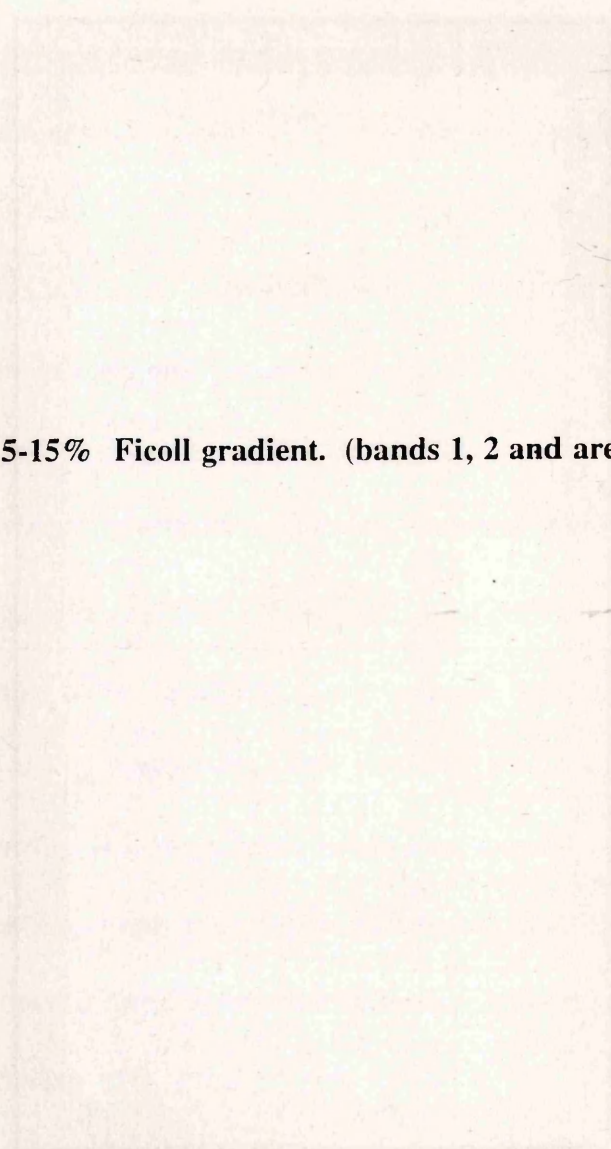


FIGURE 31.

FIV particles purified on a 5-15% Ficoll gradient. (bands 1, 2 and are identified by arrows).

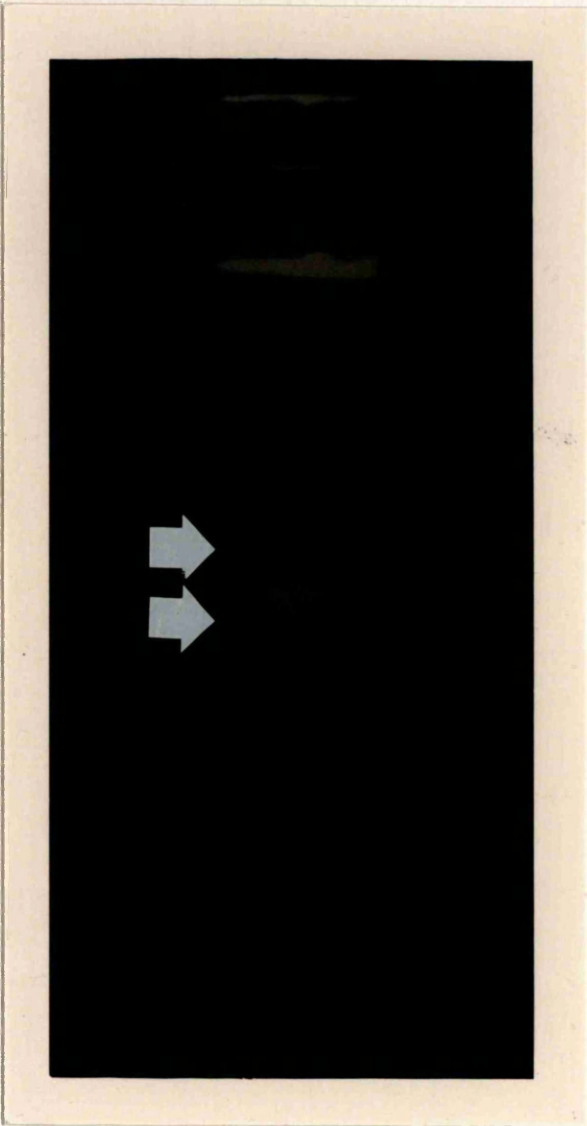


FIGURE 32.

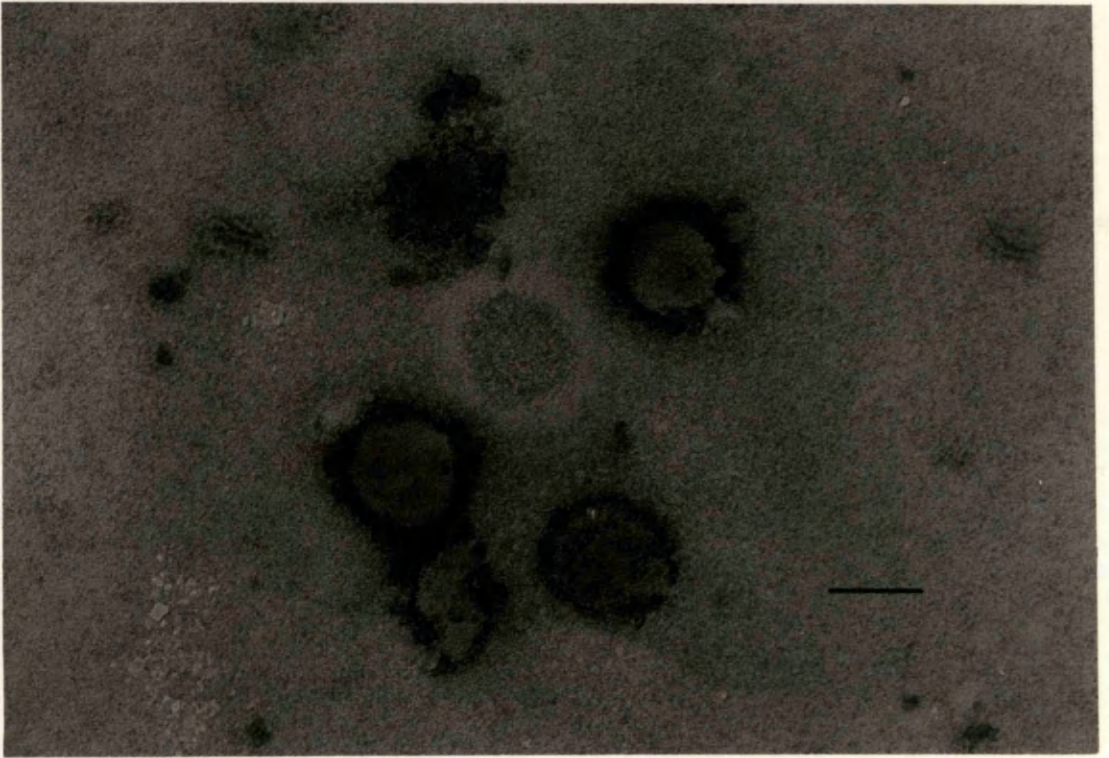
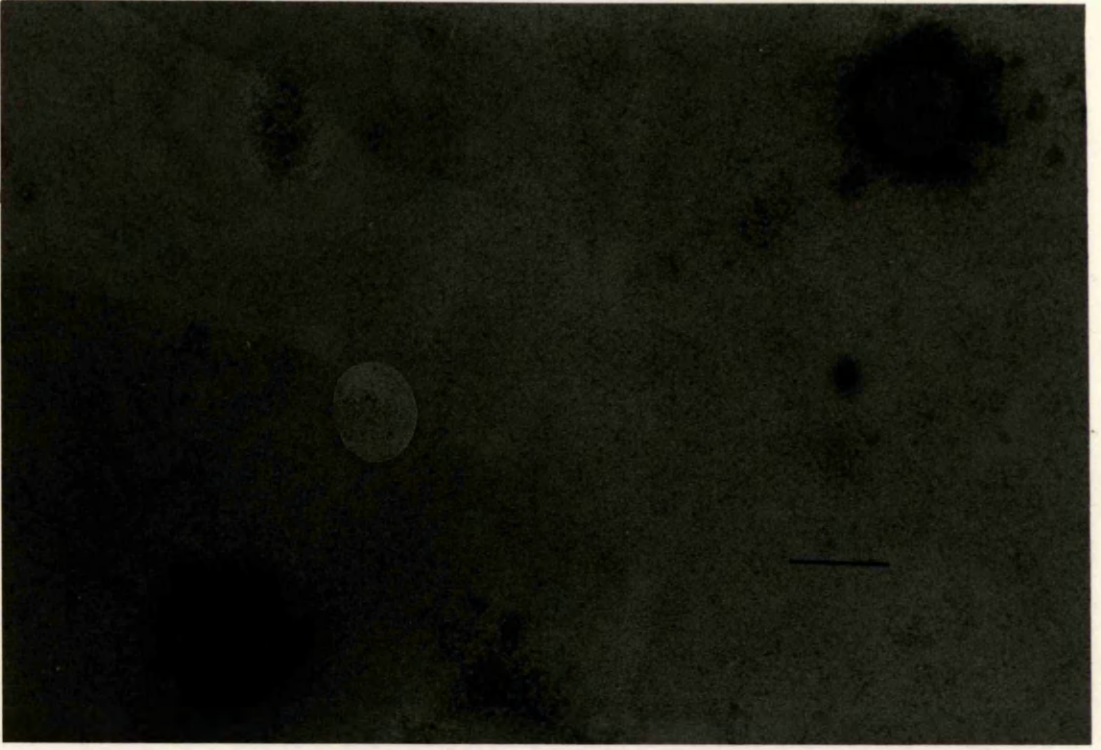
Electron micrograph of purified FIV particles banded on Ficoll gradients.

A-Top band

B-Bottom band.

Scale bar 100nm.

A



B

FIGURE 33.

A. Silver-stained polypeptide profile of purified FIV particles obtained on a 10% SDS PAGE gel.

Top band (1), bottom band (2), and molecular weight markers (M).

- Identified viral proteins (VPs).

B. Western blot of FIV particle proteins, separated by SDS PAGE (10% minigel).

FIV proteins were identified using FIV-infected cat serum.

Top band (1), bottom band (2), and molecular weight marker (M).

- Identified viral proteins (VPs).

3.3.4. SDS PAGE INVESTIGATION OF PROGENY FIV VIRUS PARTICLE PROTEINS, AND PROTEIN EXTRACTS FROM FIV PERSISTENTLY INFECTED CrFK CELL CULTURES TREATED WITH CCX.

The effect of CCX treatment on FIV polypeptide synthesis in persistently infected CrFK cell cultures, and on the polypeptide composition of the virus particles made in the presence of the drug have been investigated by SDS PAGE. Mock-infected or persistently infected CrFK cell cultures were treated with 25 μ M, or 100 μ M CCX (in medium deficient in methionine and cysteine). Three hours after drug addition, 50 μ ci/ml of 35 S methionine/ 35 S cysteine mixture was added to the tissue culture medium, and the infection allowed to proceed until 48h post drug addition. The medium was then removed from the MI and persistently infected cell cultures, then passed through a 0.22 μ filter (Acrodisc) to obtain a partially purified preparation of the cell-released virus. The material was then pelleted by centrifugation at 12000 r.p.m. in a TST41 rotor. The cell-released virus particles were then solubilized with SDS PAGE extraction buffer, and the FIV structural proteins investigated by SDS PAGE.

The SDS PAGE polypeptide profiles obtained (on a 10% gel) for materials pelleted from the tissue culture medium of MI or FIV cell cultures treated with 0 μ M, 25 μ M, or 100 μ M CCX are shown in Figure 34 A. The MI control shows the polypeptides; 175K, 100K, 70K, 66K, 58K, 38K, 32K, and 30K in material pelleted from the culture fluid. Treatment with 25 μ M CCX resulted in an increase in the amount these proteins, and in the appearance of additional proteins (150K, 54K, and 47K). Comparison of the polypeptide profile obtained from the MI drug-free control cultures and that of the MI cultures treated

with 100 μ M CCX show that several polypeptide bands were increased in intensity (70K, 66K, 58K, 54K, 52K, 48k, 47K, and 40K). While 175K, 150K, 100K, 38K, 32K, and 30K bands decreased in intensity, indicating drug-induced changes in the amounts and/or the molecular weights of proteins pelleted from MI CrFK cell culture medium.

The nature of the material pelleted from the culture medium of uninfected cells has not been investigated by electron microscopy, but probably includes very small cell membrane fragments, membrane-bound vesicles, and possibly precipitation of complexed secreted proteins. The quantitative and qualitative changes in the pattern of proteins pelleted from uninfected cells might result from CCX-induced effects on the integrity, or protein permeability of the plasma membrane, effects on the Golgi apparatus, or post-Golgi vesicles of drug-treated cells.

The proteins pelleted from the tissue culture medium of drug-free FIV-infected cell cultures are shown in Fig 34 A. The following FIV proteins were identified; p55, p24, and p17. Although not examined by electron microscopy, the material pelleted from FIV infected cell culture medium is expected to contain, predominantly, FIV particles, with a low level of contaminating cellular material. FIV particles made in the presence of 25 μ M CCX gave a polypeptide profile virtually identical to that of the drug-free control, except that the p55 protein band was increased in intensity. FIV particles made in the presence of 100 μ M CCX, however, exhibited a different polypeptide profile; the intensities of the p24, and p17 bands were dramatically reduced, while that of the p55 band was again increased. The intensities of additional protein bands of; 90K, 85K, 70K, 66K, 60K, 54K, 52K, 47K, 45K, 42K, 38K, and 36K Mwt were also increased. The identities of these proteins are unknown, but most of these bands appear to be the same as those increased in the material

pelleted from the tissue culture of MI cell cultures treated with 100 μ M CCX, suggesting that these proteins are of cellular origin. The 90K, 85K, 60k, 45K, 42K, 38K, and 36K bands however were only increased in the material pelleted from tissue culture of FIV infected cell cultures, but it is not known if these bands represent virus specific proteins.

The simplest interpretation of the observed reduction in the amount of p24, and p17 levels would be that very few virions were released from the infected cell cultures treated with 100 μ M CCX. However the observation that the amount of p55 (the precursor of p24 and p17) increased, could also be interpreted as suggesting that the virus particles were released, but that the post-release processing of p55 into p24 and p17 was impaired in the presence of CCX. Because of the low numbers of FIV particle produced it was not possible to resolve this problem by EM examination.

The SDS PAGE profile of proteins present in FIV infected or MI cell extracts is shown in Fig 34B. The polypeptide profile obtained from MI cell extract of cultures treated with 25 μ M CCX was virtually identical to that obtained from the drug-free control extract. MI cell cultures treated with 100 μ M CCX showed an altered polypeptide profile. Bands of 70K, 66K, 58K, and 23K were increased in intensities, while the 29K band was decreased in intensity. The 70K and 66K bands appear to correspond to the 70K and 66K bands pelleted from the cell culture medium of MI cells treated with 100 μ M CCX (Fig 34A). The polypeptide patterns of MI and FIV infected cell extracts, grown in the absence of CCX, differ in the intensities of only two bands; (39K and 30K were considerably reduced in the intensity in the MI cell extracts), but FIV proteins with these molecular weights have not been previously reported. Protein extracts from infected cell cultures treated with 25 μ M band of 55K tentatively identified as FIV p55 was increased in intensity. The material

CCX gave a polypeptide pattern virtually identical to the FIV control except that the intensity of the novel 39K band was reduced.

The polypeptide profiles obtained from protein extracts of the FIV infected cell cultures treated with 100 μ M CCX was markedly different from the FIV infected control culture. Bands of 97K, 80K, 68K, 62K, 55K, 52K, 50K, 44K, 42K, 41K, 38K, 37K, 34K, 31K, 30K, 27K, 24k, 22K, 21K, and 15K, were all increased in intensity; while bands of; 175K, 150K, 100K, 70K, 67K, 48K, 40K, 29K, 19K, and 18K were decreased in intensity. However, with the exception of the 55K and the 41K bands none of these proteins have molecular weights corresponding to those of known FIV proteins.

Comparison of the polypeptide profiles of the pelleted FIV particles (Figure 34A) and FIV infected cell extracts (Figure 34B) treated with 100 μ M CCX shows that the 55K band, (tentatively identified as the FIV p55 protein) increased in intensity both in the pelleted material from tissue culture supernatant and in the infected cell extracts. Suggesting impairment of both particle assembly and post-release processing of p55. As expected the cleavage products of p55 (p24 and p17) were not detected in the infected cell extracts as these arise from post-release cleavage of p55.

It is concluded from the data in Fig 34 that treatment of FIV infected CrFK cell cultures with 25 μ M CCX had little effect on protein synthesis in infected cells, and yielded virus particles whose polypeptide composition was virtually identical to that made in the absence of the drug. Treatment with 100 μ M CCX, however resulted in a dramatic change in the polypeptide profile of the infected cell extract; a number of polypeptide bands appeared to increase in intensity in infected cell extracts only (among these polypeptides the band of 55K tentatively identified as FIV p55 was increased in intensity). The material

pelleted from tissue culture medium from infected cell cultures treated with 100 μ M CCX also had an increased level of p55 and reduction in the amount of p24, and p17. The data might suggest a reduction in the number of virus particles produced in cultures treated with 100 μ M CCX or an increase in the extracellular release of a set of proteins including p55.

To further investigate the effect of CCX on FIV glycoprotein synthesis; MI and FIV infected cell cultures were treated with 5 μ M monensin, or 2 μ g/ml tunicamycin (Fig 34). The sodium ionophore, monensin is well-known to inhibit maturation of glycoproteins in the Golgi apparatus (Gareth *et al* 1983), while tunicamycin inhibits the N-linkage of the initial set of core sugars to the native precursor protein, at a stage prior to its translocation to the Golgi apparatus, and while the protein is resident in the lumen of the ER.

There was no evidence in the SDS PAGE polypeptide profiles for the presence of the FIV env, glycoprotein non-glycosylated precursor gp(160), nor any evidence of partially-processed precursor glycoproteins packaged into the virus particles made in the presence of monensin or tunicamycin. Similarly, the gp160 or partially processed products were not detected in the SDS PAGE polypeptide profiles obtained from extracts of FIV infected cell cultures grown in the presence of monensin or tunicamycin. Furthermore, while monensin and tunicamycin treatment had no effect on the level of p55 in infected cell cultures or in virus particles, treatment with either of the compounds reduced the intensity of p24, and p17 bands in the pelleted virus particles, suggesting that fewer virus particles were produced in the presence of the glycosylation inhibitors.

Treatment of MI cell cultures with monensin had no significant effect on the SDS PAGE polypeptide pattern, but tunicamycin treatment resulted in an increase in the

intensities of 3 other proteins (66K, 38K and 18k), thought to be host cell glycoprotein precursors (Figure 34B).

Previous studies in which the effect of CCX and monensin treatments on the HSV or SFV infected cell cultures were compared, have shown broad similarities between the effect of CCX and monensin and suggest that CCX affects glycosylation at the level of processing in the Golgi apparatus (Dargan et al., 1992). The studies reported here, however, provide no information on FIV glycosylation processing in cells treated with CCX, monensin, or tunicamycin.

To further investigate the effect of CCX on FIV glycoprotein processing, MI and FIV infected cell were labelled with ^{14}C -glucosamine, and the virus proteins immunoprecipitated with an FIV infected cat serum. The ^{14}C -glucosamine labelled proteins were electrophoresed on the same SDS PAGE gel with ^{35}S -methionine/cysteine labelled proteins to allow identification of the ^{35}S -labelled glycoproteins. As before, material pelleted from supernatant culture fluid and infected cell extracts were investigated. In this experiment 7.5% acrylamide gels were used to enhance the resolution of the FIV glycoproteins (gp120, gp160)

The effect of 25 μM , and 100 μM CCX on the proteins pelleted from the tissue culture medium of MI, and FIV infected, cell cultures is shown in Fig 35. Four, high-molecular weight bands labelled with ^{14}C glucosamine were immunoprecipitated non-specifically by the FIV infected cat serum in the material pelleted from drug-free MI CrFK cell cultures. The intensities of these bands were markedly reduced by treatment of the cultures with 25 μM CCX, and were not detectable when the cultures were treated with

FIGURES 34 A.

Polypeptide profiles obtained on a 10% SDS PAGE gel of ^{35}S -methionine/cysteine labelled proteins pelleted from the culture medium of MI (lanes 4, 5, 6, 7, and 9), or FIV-infected (lanes 1, 2, 3, 8, and 10) CrFK cells.

Cells were either drug-free (lanes 1 and 4), or treated with 25, or 100 μM CCX (lanes 2 and 5; 3 and 6 respectively), 5 μM monensin (lanes 7 and 8) or 2 $\mu\text{g/ml}$ tunicamycin (lanes 9 and 10).

The location of the molecular weight markers (M) (non-radiolabelled) resolved on the same gel, have been transferred from the dried gel.

- ▲ Identified viral proteins.
- Protein bands of unknown origin.

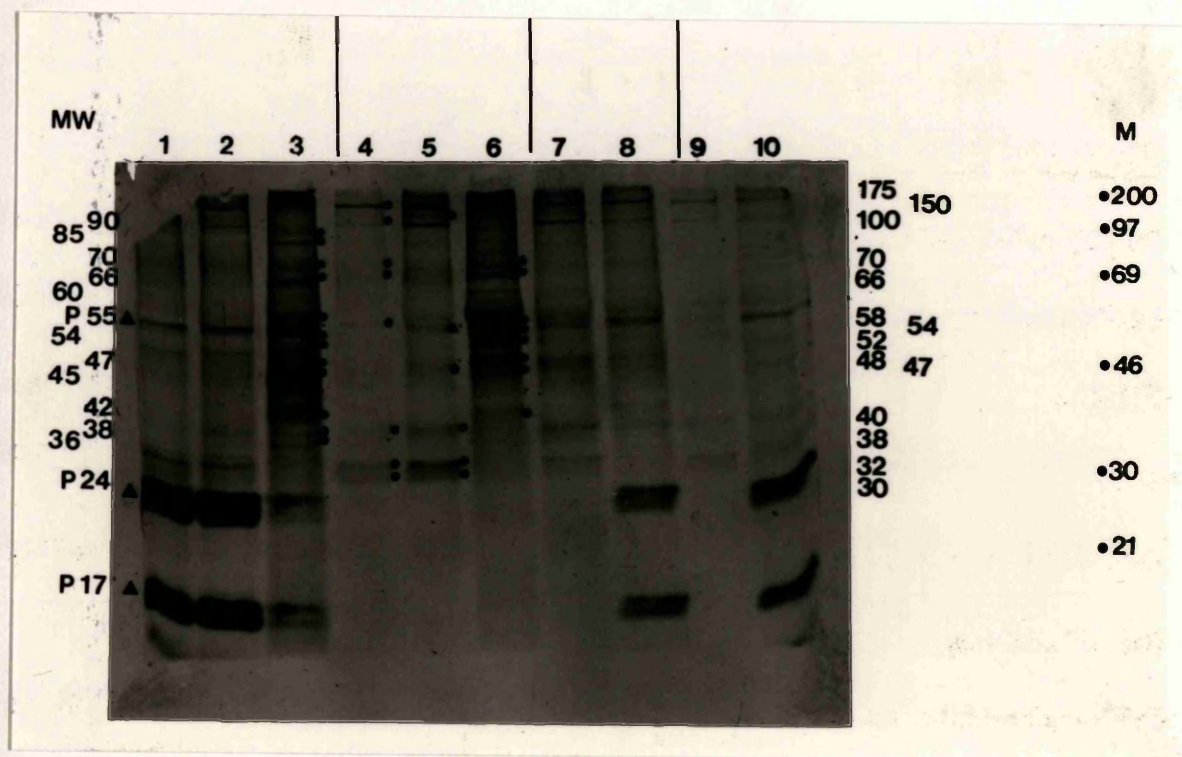


FIGURE 34 B.

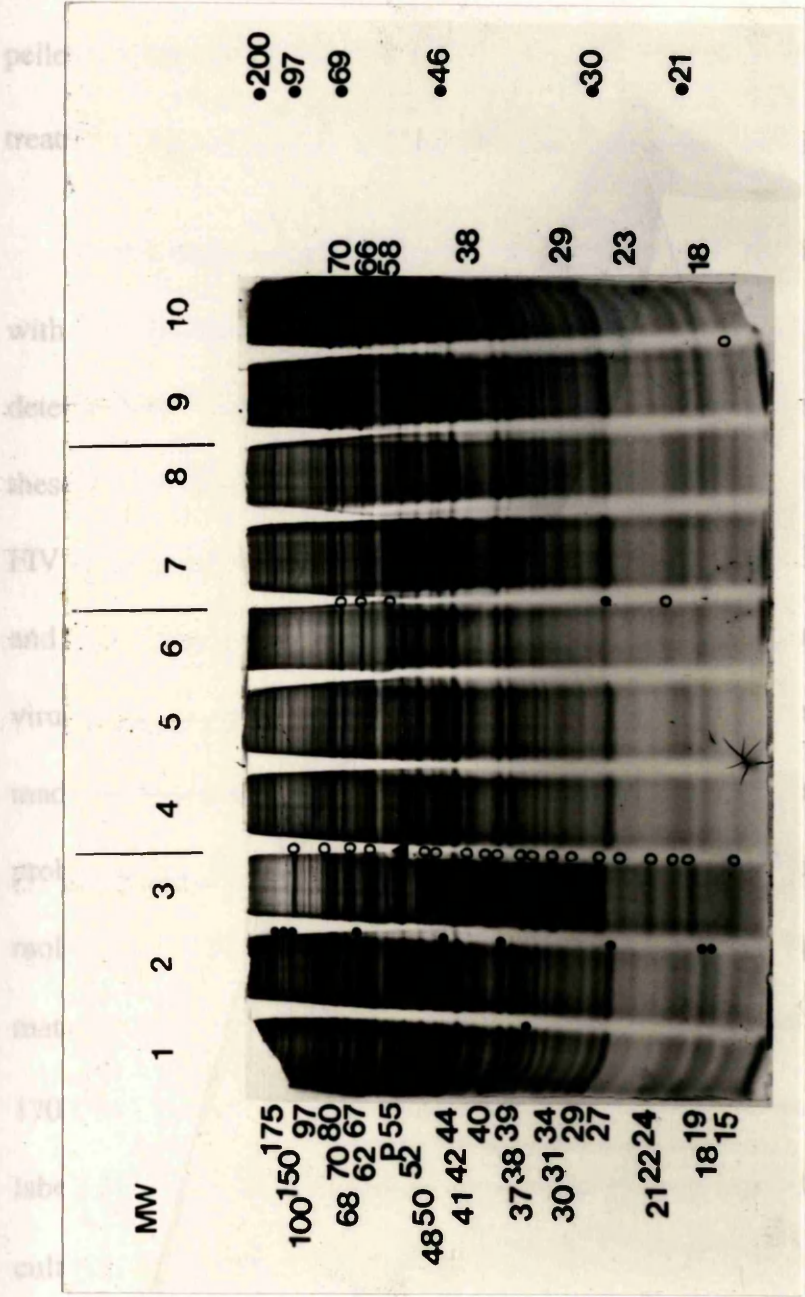
Polypeptide profiles obtained on a 10% SDS PAGE gel of ^{35}S -methionine/cysteine labelled proteins in the extracts from MI (lanes 4, 5, 6, 7, and 9), or FIV-infected (lanes 1, 2, 3, 8, and 10) CrFK cells.

Cells were either drug-free (lanes 1 and 4) or treated with 25, or 100 μM CCX (lanes 2 and 5; 3 and 6 respectively), 5 μM monensin (lanes 7 and 8), or 2 $\mu\text{g/ml}$ tunicamycin (lanes 9 and 10).

The location of molecular weight markers (M) (non-radiolabelled) resolved on the same gel have been transferred from the dried gels.

- ▲ Identified virus proteins.
- Bands decreasing in intensity with increasing drug concentration.
- Bands increasing in intensity with increasing drug concentration.

100µM CCX, suggesting that the glycosylation or secretion of these proteins was altered by CCX treatment (Fig 35A). The SDS PAGE polypeptide profile of ³⁵S-labelled material



not affected by 25µM CCX (Figure 35A).

infected cell cultures labelled (Fig 35B). Only three bands are

the infected-cell cultures, one of immunoprecipitated by the anti

FIV gp120 (barely detectable)

present in equimolar amount in

g) shows that in FIV particles

r amounts than gp120. This is

tion in the number of gp120

gp41 were not detected in the

only 25µM CCX, although the

intensity. No ¹⁴C glucosylation

the medium of the infected cell

Gp41 and gp120 bands were not detected in the drug-free material pelleted from the infected cell cultures labelled with ³⁵S-methionine/cysteine. A band in the region of 41K increased in a drug-dose dependent manner, however it is not clear whether this is gp41 or whether it is a host cell protein that does not behave in the same way in M1 cell cultures.

100 μ M CCX, suggesting that the glycosylation or secretion of these proteins was altered by CCX treatment (Fig 35A). The SDS PAGE polypeptide profile of 35 S-labelled material pelleted from the culture medium of MI cell cultures was not affected by 25 μ M CCX treatment but was altered when treated with 100 μ M CCX (Figure 35A).

The material pelleted from the supernatant of FIV infected cell cultures labelled with 14 C-glucosamine or 35 S-methionine/cysteine is shown in Fig 35B. Only three bands are detectable in 14 C-glucosamine labelled material from drug-free infected cell cultures, one of these is a 170K host cell polypeptide band [non-specifically immunoprecipitated by the anti FIV serum (Figure 35B)], while the other two correspond to FIV gp120 (barely detectable), and gp41. It is expected that gp120 and gp41 should be present in equimolar amount in virus particles, scrutiny of the gel (14 C glucosamine labelling) shows that in FIV particles made in the absence of the drug, gp41 was present in greater amounts than gp120. This is probably due to pelleting of the virus resulting in a reduction in the number of gp120 molecules present on the virion envelope. Gp120, and gp41 were not detected in the material pelleted from the infected cell cultures treated with only 25 μ M CCX, although the 170K host protein band was detected and slightly reduced in intensity. No 14 C glucosamine labelled protein was detected in the material pelleted from the medium of the infected cell cultures treated with 100 μ M CCX.

Gp41 and gp120 bands were not detected in the drug-free material pelleted from the infected cell cultures labelled with 35 S-methionine/cysteine. A band in the region of 41K increased in a drug-dose dependent manner, however it is not clear whether this is gp41 or whether it is a host cell protein that does not behave in the same way in MI cell cultures.

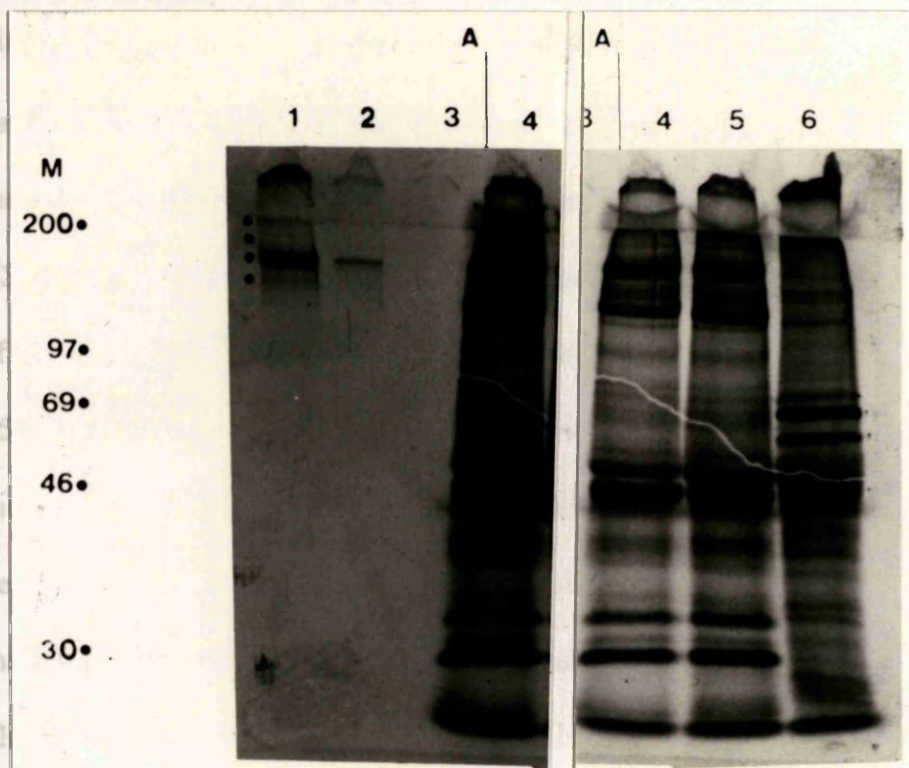
FIGURE 35.

Polypeptide profiles obtained on a 7.5% SDS PAGE gel of proteins pelleted from MI (A), or FIV-infected (B) CrFK cells. Cells were labelled with either ^{35}S -methionine/cysteine (lanes 4, 5, and 6; Figures A and B), or ^{14}C -glucosamine (lanes 1, 2, and 3; Figures A and B).

Cells were either drug-free (lanes 1 and 4), or treated with 25, or 100 μM CCX (lanes 2 and 5; 3 and 6 respectively, Figures A and B).

The location of the molecular weight markers (M) (non-radiolabelled) resolved on the same gel, have been transferred from the dried gel.

- ▲ Identified viral proteins.
- Protein bands of unknown origin.



100μM CCX, several

own whether any of

ins. The following

asing concentration

5K, 44K, 43K, 41K,

24K decreased in

or glycoprotein is

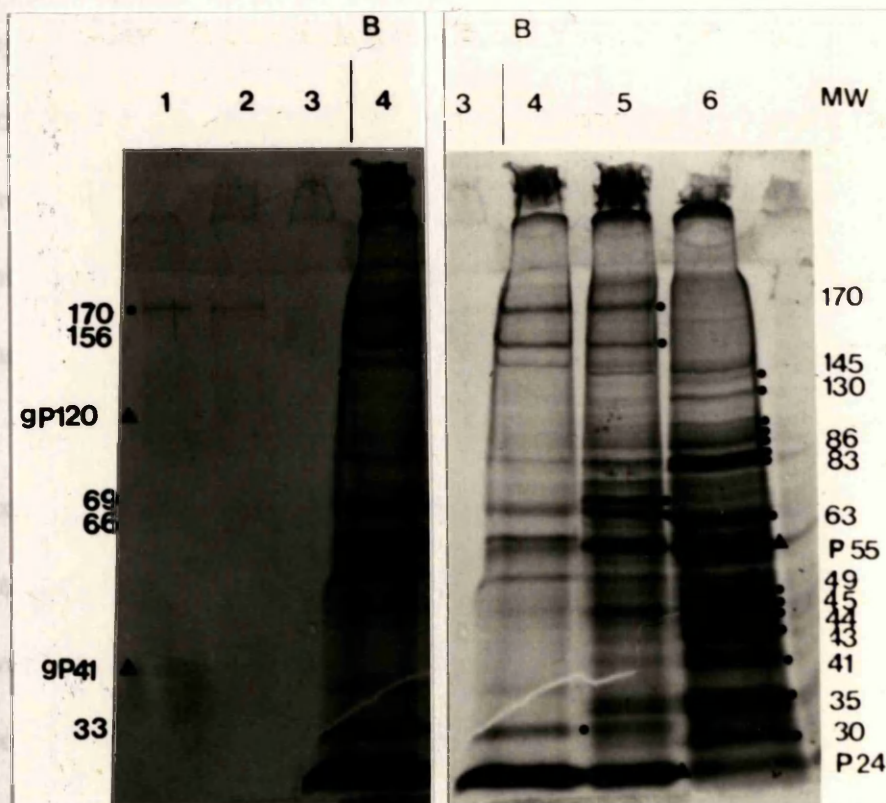
were increased in

road band of 35 S

the bands represent a

partially glycosylated form of gp120, it is difficult without data from either a western blot,

or immunoprecipitation reactions to identify, which if any of these 35 S-labelled bands



M

•200

•97

•69

•46

•30

Similarly in the material pelleted from cultures treated with 25 μ M, or 100 μ M CCX, several bands in the region of 120K band increased in intensity, but it is not known whether any of these bands are the FIV gp120 or whether they represent host cell proteins. The following ³⁵S-methionine/cysteine labelled bands increased in intensity with increasing concentration of CCX; 145K, 130K, 115K, 106K, 97K, 86K, 83K, 63K, 55K, 49K, 45K, 44K, 43K, 41K, 35K, and 30K, while bands of 170K, 156K, 69K, 66K, 33K, and 24K decreased in intensity. Stephens *et al.*, (1991) have shown that the FIV precursor glycoprotein is decorated with core sugars. Bands of 115K, 106K, and 97K which were increased in intensity with increasing CCX concentration co-run with the broad band of ¹⁴C glucosamine-labelled gp120, and it is possible that one or more of these bands represent a partially glycosylated form of gp120. It is difficult without data from either a western blot, or immunoprecipitation reactions to identify, which if any of these ³⁵S-labelled bands represent the viral glycoproteins. A band of 55K was increased in intensity, in the polypeptide profile of material pelleted from drug-free FIV infected cell cultures, with increasing concentrations of CCX. This band co-runs with a host band of about the same molecular weight in the tissue culture medium of MI cell cultures. However the intensity of the MI band decreased with increasing drug concentration.

Figure 36 shows the SDS PAGE polypeptide profile of MI and FIV infected cell extracts obtained from cultures labelled with ¹⁴C-glucosamine or ³⁵S-methionine/cysteine and treated with increasing concentration of CCX. The ¹⁴C-glucosamine labelled proteins were immunoprecipitated using an FIV-infected cat serum. The ³⁵S-labelled proteins were not immunoprecipitated. As expected no ¹⁴C-glucosamine labelled MI cell glycoproteins were detected after immunoprecipitation (Fig 36A). Only one ³⁵S-labelled band (60K) was

FIGURE 36.

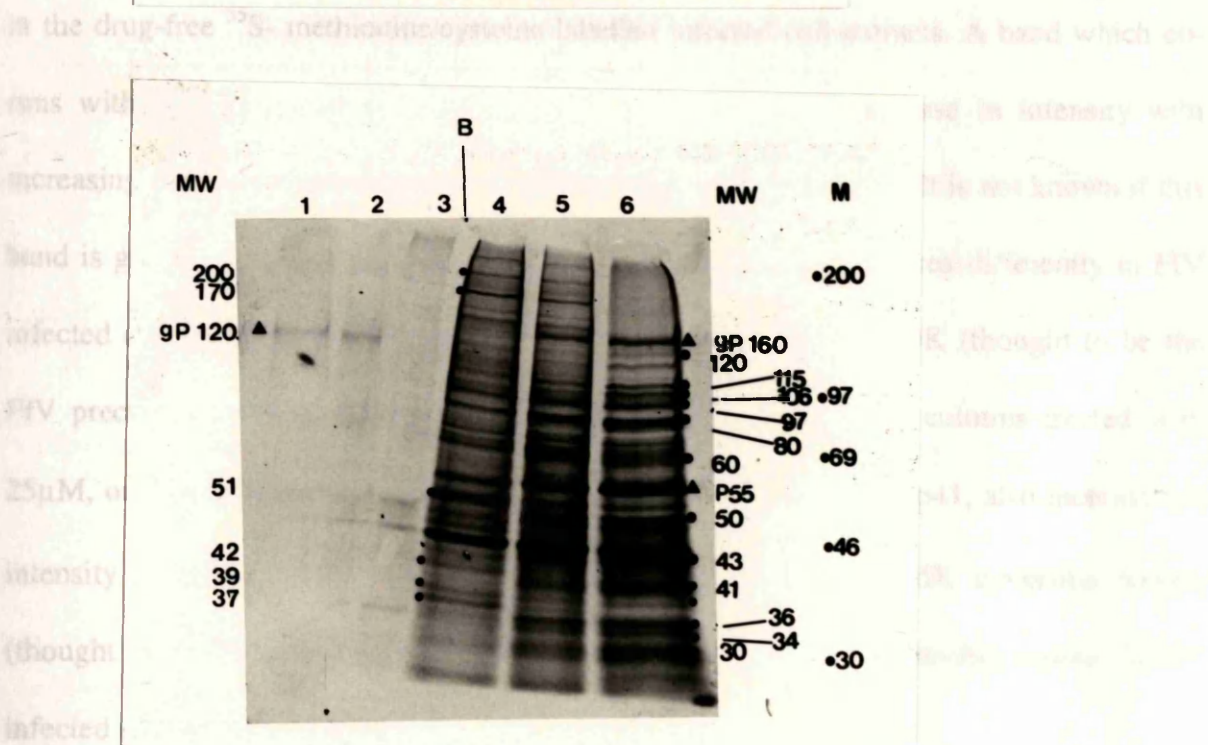
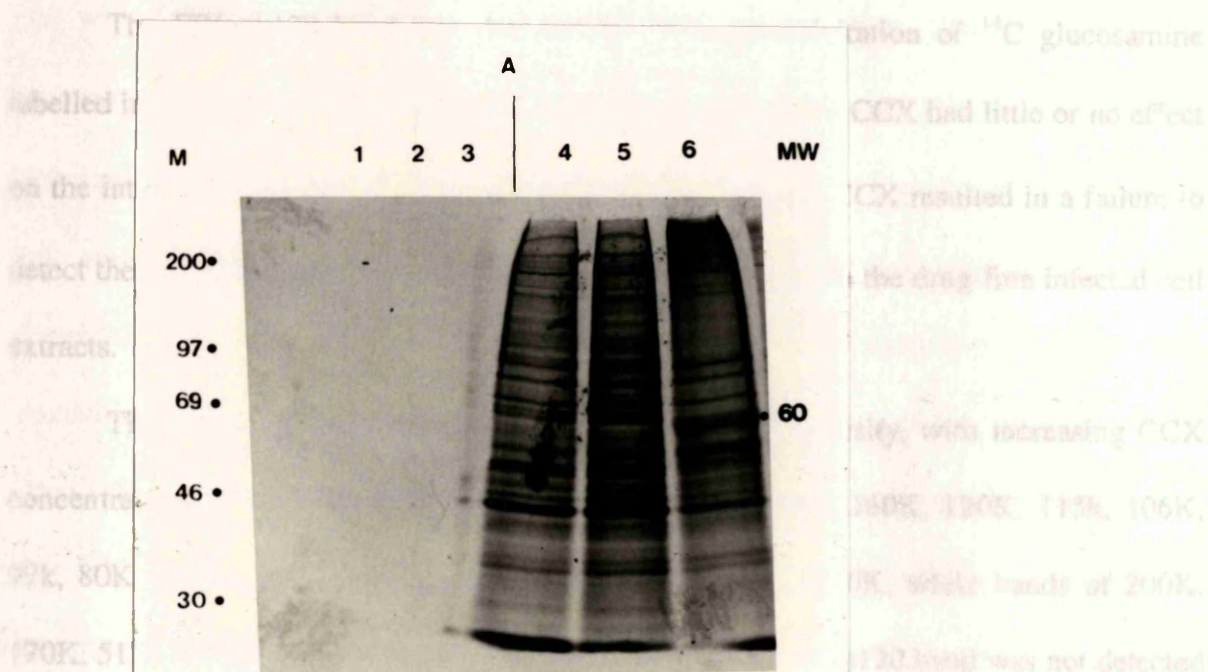
Polypeptide profiles obtained on a 7.5% SDS PAGE gel of extracts from MI (A), or FIV-infected (B) CrFK cells. Cells were labelled with either ^{35}S -methionine/cysteine (lanes 4, 5, and 6 Figures A and B) or ^{14}C -glucosamine (lanes 1, 2, and 3 Figures A and B).

Cells were either drug-free (lanes 1 and 4) or treated with , 25, or 100 μM CCX (lanes 2 and 5; 3 and 6 respectively, Figures A and B)

The location of molecular weight markers (M) (non-radiolabelled) resolved on the same gel have been transferred from the dried gel.

- ▲ Identified virus proteins.
- Protein bands of unknown origin.

increased in intensity in cell extracts from cells treated with 100 μ M CCX. Most, but not all other bands were reduced in intensity.



In conclusion, CCX treatment of infected cells resulted in a decrease in the amount of sugar-labeled gp120, suggesting that the amount of gp120 released from the cells was reduced.

increased in intensity in cell extracts from cells treated with 100 μ M CCX. Most, but not all other bands were reduced in intensity.

The FIV gp120 band was detected by immunoprecipitation of 14 C glucosamine labelled infected cell extracts (Fig 36B). Treatment with 25 μ M CCX had little or no effect on the intensity of the gp120 band, but treatment with 100 μ M CCX resulted in a failure to detect the gp120 band. The FIV Gp 41 band was not detected in the drug-free infected cell extracts.

The following bands were observed to increase in intensity, with increasing CCX concentration, in the extracts from 35 S-labelled infected cells; 160K, 120K, 115k, 106K, 97k, 80K, 60K, 55K, 50K, 43K, 41K, 36K, 36K, 34K, and 30K, while bands of 200K, 170K, 51K, 42K, 39K, and 37K were decreased. Although the gp120 band was not detected in the drug-free 35 S- methionine/cysteine labelled infected cell extracts. A band which co-runs with 14 C- glucosamine labelled gp120 was observed to increase in intensity with increasing drug concentrations in the 35 S-labelled extracts (Fig 36B). It is not known if this band is gp120, or whether it represents a host cell protein that behaves differently in FIV infected cell cultures. A higher molecular weight band of about 160K (thought to be the FIV precursor gp 160) increased in intensity in cell extracts from cultures treated with 25 μ M, or 100 μ M CCX (Fig 36B). Similarly a band, thought to be gp41, also increased in intensity in extracts from drug-treated cell cultures. The band of 55K molecular weight (thought to be FIV p55) also increased in intensity in a drug-dose dependent manner in FIV infected cell extracts (Fig 36B).

In conclusion, CCX treatment of infected cell cultures resulted in a reduction in the amount of sugar-labelled gp120, suggesting that either the glycosylation or the synthesis of

gp120 was impaired. If, however, the identification of ^{35}S -labelled gp120 and gp160 is correct then these partially glycosylated proteins (poorly labelled with ^{14}C -glucosamine, but not with ^{35}S -methionine) accumulate in the cells treated with $100\mu\text{M}$ CCX. The accumulation of gp160 would imply that cleavage of gp160 into gp120 and gp41 was impaired by treatment with $100\mu\text{M}$ CCX. The p55 protein band increased in FIV infected cell extracts in a drug-dose dependent manner, this protein also increased in a dose dependent manner in virus particles pelleted from the tissue culture medium of drug-treated infected cell cultures. The p24 band decreased in intensity in the material pelleted from infected cell cultures treated with the drug. It is concluded from this set of the data that FIV assembly, release and possibly the post-release maturation of FIV are impaired by treatment with $100\mu\text{M}$ CCX.

3.4. INVESTIGATION OF THE EFFECT OF TRITERPENOID COMPOUNDS ON THE REPLICATION OF BOVINE HERPESVIRUS TYPE 1 (BHV-1)

Among the herpesviruses, the anti-HSV-1 activity of CCX has been most extensively studied, however the herpesvirus which exhibited the highest degree of sensitivity to CCX treatment was BHV-1 (Galt et al, 1990).

3.4.1. THE EFFECT OF CCX TREATMENT ON THE 24H INFECTIOUS BHV-1 YIELDS FROM MDBK CELLS IN CULTURE.

Figure 37, shows the effect of CCX treatment on the 24h infectious yield of BHV-1 from MDBK cell cultures. 1×10^6 cells/30mm culture plate were infected with BHV-1 at m.o.i. of 5 p.f.u./cell. After a 1h virus absorption period, the plates were washed and overlaid with medium containing increasing concentrations of CCX. The infected cell cultures were incubated at 37°C, for 24h, after which the CA and the CR virus yields were harvested. Drug-free, infected cell cultures yielded 1.065×10^8 p.f.u./ml and 4.59×10^8 p.f.u./ml in the CR and CA fractions respectively. Treatment with 100µM, 200µM, or 300µM CCX gave 200, 2.45×10^4 , and 4.73×10^5 fold reductions in the infectivity of CR fractions respectively, while the CA infectivity was reduced by 26.2, 1.25×10^3 , and 6.95×10^4 fold respectively. Thus, while infectivity decreased in a more or less linear fashion with increasing drug concentrations, CR infectivity decreased at a faster rate than CA infectivity.

This suggests either a virucidal activity of the drug on extracellular virus particles, or impairment of the release of virus into the extracellular fluid.

Concentration of the drug inhibiting BHV-1 virus yield by 25%, 50%, or 90% (ED25, ED50-ED 90).

Compound	ED25	ED50	ED90
CCX	20uM*	31.5uM*	62uM*

* Numbers calculated based on the total virus yield i.e. cell released plus cell associated.

YIELDS FROM MDBK CELLS IN CULTURE

Figure 37, shows the effect of CCX treatment on the 24h infectious yield of BHV-1 from MDBK cell cultures. 1×10^6 cells/50mm culture plate were infected with BHV-1 at a m.o.i. of 5 p.f.u./cell. After a 1h virus adsorption period, the plates were washed and overlaid with medium containing increasing concentrations of CCX. The infected cell cultures were incubated at 37°C , for 24h, after which the CA and the CR virus yields were harvested. Drug-free, infected cell cultures yielded 1.06×10^6 p.f.u./ml and 4.39×10^6 p.f.u./ml in the CR and CA fractions respectively. Treatment with 100uM, 200uM, or 300uM CCX gave 200, 2.45×10^4 , and 4.73×10^5 fold reductions in the infectivity of CR fractions respectively, while the CA infectivity was reduced by 26.2, 1.35×10^2 , and 6.95×10^2 fold respectively. Thus, while infectivity decreased in a more or less linear fashion with increasing drug concentrations, CR infectivity decreased at a faster rate than CA infectivity. This suggests either a virucidal activity of the drug on extracellular virus particles, or impairment of the release of virus into the extracellular fluid.

3.4.2. **Figure. 37**
THE EFFECT OF CCX TREATMENT ON THE 24H
INFECTIOUS BHV-1 YIELDS FROM MDBK CELLS

In order to investigate whether the triterpenoid compounds (CCX, CBX, or GL) have virucidal activity against BHV-1, suspensions of BHV-1 particles prepared in medium containing different concentrations of each drug were incubated, at either 4°C or 37°C, for 24h after which, the remaining infectivity was titrated. Table 7, shows that treatment with 150µM CCX, 150µM CBX, or 750µM GL had a small virucidal activity at 4°C (2.2, 2.7, and 3.3 fold reductions in infectivity respectively). Doubling the concentrations of the drug had little or no effect on the virucidal activity. Incubation at 37°C had little effect on the virucidal activity observed with 150µM, 150µM CBX, or 750µM CCX. However, doubling the drug concentrations in suspensions incubated at 37°C increased the magnitude of the virucidal effect (3.3, 4.1, and 4.2 fold).

It is concluded, that while virucidal activity (2 fold) can be detected in suspension treated with 150µM CCX at 37°C, this is insignificant compared to the 29.3 fold difference in infectivity between the CR and CA action obtained from infected cells treated with 300µM CCX. Thus the data suggest that CCX treatment results in impairment of BHV-1

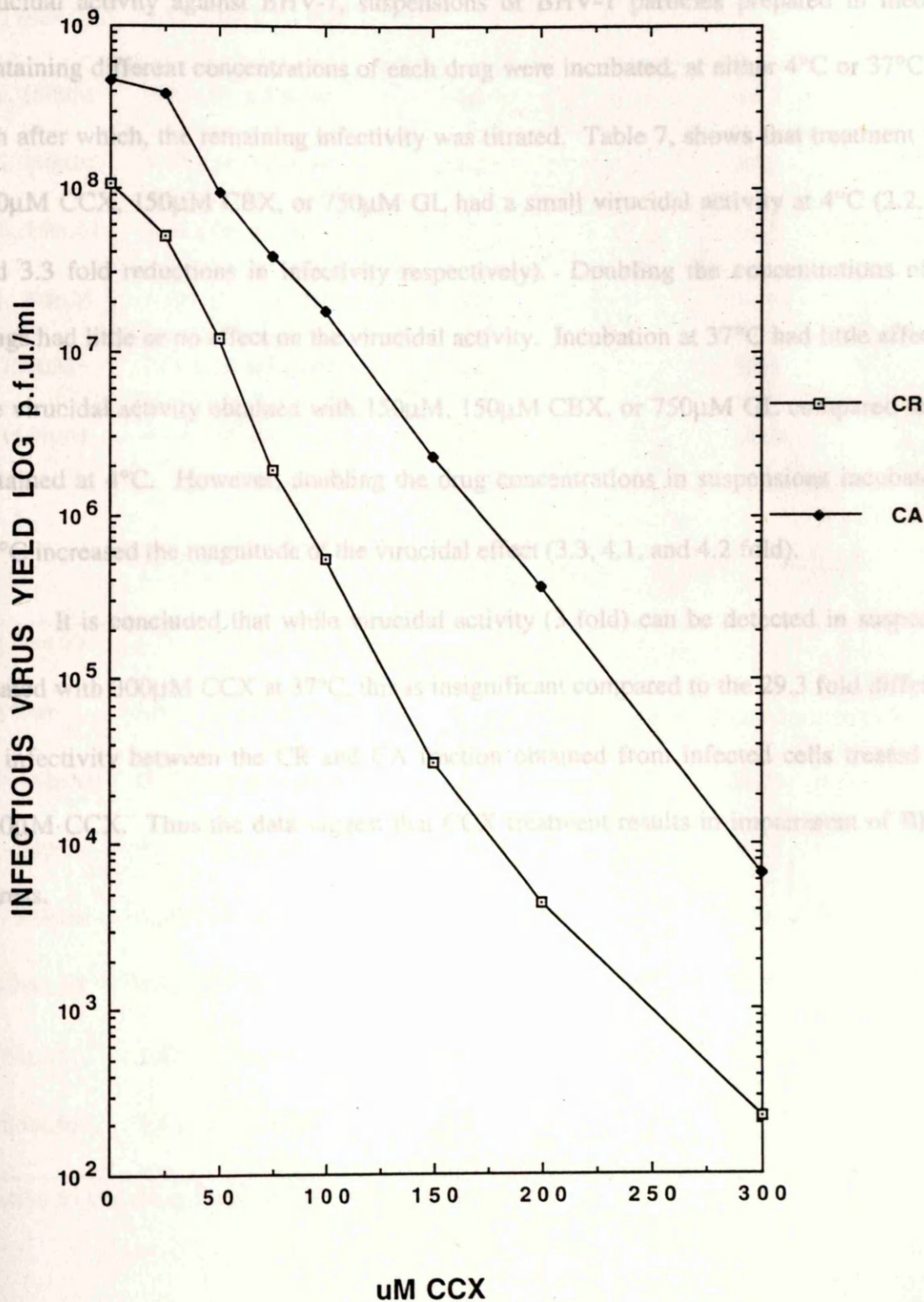


TABLE 7. BHV-1: VIRUCIDAL ACTIVITY OF CCX, CBX, AND GL
DETERMINED AFTER 24H INCUBATION AT 4°C (A) OR

3.4.2. INVESTIGATION OF THE VIRUCIDAL ACTIVITY OF CCX, CBX, AND

GL AGAINST BHV-1.

In order to investigate whether the triterpenoid compounds (CCX, CBX, or GL) have virucidal activity against BHV-1, suspensions of BHV-1 particles prepared in medium containing different concentrations of each drug were incubated, at either 4°C or 37°C, for 24h after which, the remaining infectivity was titrated. Table 7, shows that treatment with 150µM CCX, 150µM CBX, or 750µM GL had a small virucidal activity at 4°C (2.2, 2.7, and 3.3 fold reductions in infectivity respectively). Doubling the concentrations of the drugs had little or no effect on the virucidal activity. Incubation at 37°C had little effect on the virucidal activity obtained with 150µM, 150µM CBX, or 750µM GL compared to that obtained at 4°C. However, doubling the drug concentrations in suspensions incubated at 37°C increased the magnitude of the virucidal effect (3.3, 4.1, and 4.2 fold).

It is concluded that while virucidal activity (3 fold) can be detected in suspension treated with 300µM CCX at 37°C, this is insignificant compared to the 29.3 fold difference in infectivity between the CR and CA fraction obtained from infected cells treated with 300µM CCX. Thus the data suggest that CCX treatment results in impairment of BHV-1 egress.

TABLE 7. BHV-1: VIRUCIDAL ACTIVITY OF CCX, CBX, AND GL DETERMINED AFTER 24H INCUBATION AT 4°C (A), OR 37°C (B).

A

DRUG	TITRE	FOLDREDUCTION	% REDUCTION*
Drug free 0h	1.71 x 10 ⁹ p.f.u./ml	—	—
Drug free	1.18 x 10 ⁸ p.f.u./ml	—	—
CCX, 150µM	5.4 x 10 ⁷ p.f.u./ml	2.2	54.3
CCX, 300µM	5.47 x 10 ⁷ p.f.u./ml	2.2	53.6
CBX, 150µM	4.34 x 10 ⁷ p.f.u./ml	2.7	63.2
CBX, 300µM	5.41x 10 ⁷ p.f.u./ml	2.2	54.15
GL, 750µM	3.52 x 10 ⁷ p.f.u./ml	3.3	70.2
GL, 1500µM	3.15 x 10 ⁷ p.f.u./ml	3.7	26.7

B

DRUG	TITRE	FOLD REDUCTION	% REDUCTION*
Drug free 0h	1.71 x 10 ⁹ p.f.u./ml		
Drug free	5.83 x 10 ⁸ p.f.u./ml	—	—
CCX- 150µM	3.75 x 10 ⁸ p.f.u./ml	1.5	35.7
CCX- 300µM	1.74 x 10 ⁸ p.f.u./ml	3.3	70.15
CBX- 150µM	1.89 x 10 ⁸ p.f.u./ml	3.1	67.6
CBX-300µM	1.43 x 10 ⁸ p.f.u./ml	4.1	75.5
GL-750µM	1.87 x 10 ⁸ p.f.u./ml	3.1	67.9
GL-1500µM	1.4 x 10 ⁸ p.f.u./ml	4.2	7

*** Relative to the drug-free titre after 24h incubation.**

3.4.3. THIN-SECTION TRANSMISSION ELECTRON MICROSCOPE

STUDIES OF BHV-1 INFECTED AND MI MDBK CELL CULTURES

TREATED WITH INCREASING CONCENTRATIONS OF CCX.

To further investigate the effect of CCX on BHV-1 replication, monolayers of MDBK cells on 30mm tissue culture plates were either MI, or infected with BHV-1 at 5 p.f.u./cell. After the virus absorption period, cell cultures were overlaid with either drug-free medium, or medium containing, 50µM, 150µM, 200µM, or 300µM CCX. At 24h PI, the cell cultures were fixed with gluteraldehyde and prepared for transmission electron microscopy (Material and Methods section 2.2.6.4.2-3.).

Forty cell sections were scored for the numbers of DNA containing, empty, or partially filled nucleocapsids within the nucleus or cytoplasm; the numbers of enveloped or non-enveloped virus in the cytoplasm, and the numbers of virus particles in the extracellular matrix. The effect of CCX treatment on the number of total, empty, and core containing virus particles and number of enveloped virus particles is shown in table 8. The effect of increasing CCX concentration on; the percentage of cell sections containing virus particles; the percentage of empty and DNA containing particles; the percentage of enveloped virus particles in the cytoplasm; and the percentage of virus particles present in the extracellular medium shown in table 9. The results in column 2 (table 8) show a general reduction in the total number of capsids (core containing, and empty) with increasing concentration of CCX. The percentage of cell sections containing virus particles was reduced (92.5%, 87.5%, 85%, 22%, and 0% for cultures treated with 0µM, 50µm, 150µm, 200µm, and 300µM CCX respectively) (column 2 table 9), while the average number of virus particles/section were 24.5, 27.1, 9.5, 0.3, and 0 respectively (column 3,

table 9). Thus, generally fewer virus particles were assembled in each infected cell. The data in column 4 (table 9), shows that the percentage number of empty capsids structures was unaffected by treatment with up to 150 μ M CCX, although the percentage rose from around 20% to 82% in cells treated with 200 μ M CCX. In the drug-free cultures all of the virus particles found in the cytoplasm were enveloped (column 8, table 9). With increasing drug concentration, no consistent effect on envelopment of the virus is observed (column 8, table 9). The data in column 9 (table 9) shows that percentage of extracellular viruses per cell section for the cultures treated with 50 μ M, 150 μ M, 200 μ M, and 300 μ M CCX were 24.5%, 31.6%, 3.57%, and 2.27%, of the drug-free control respectively. The data in table 9 column 5 shows that treatment with increasing concentration of CCX did not have any significant effect on the percentage of cored particles. Also the data in table 8 columns, 5 and 6, shows that the number of virus particles exhibiting a DNA core, present in the cytoplasm decreased at a faster rate than the number of virus particles present in the nucleus, implying that virus release from the nucleus through the nuclear membrane was also inhibited by the drug. It is concluded that CCX treatment inhibits the assembly of nucleocapsids, and release of the virus particles from the nucleus.

TABLE. 8. The effect of CCX treatment on number of empty and cored BHV-1 particles, number of enveloped particles in cytoplasm, and number of viruses in extracellular medium.

μ M CCX	capsids counted	empty capsid structures	core containing structures	enveloped particles in cytoplasm	nonenveloped particles in cytoplasm	sections containing virus particles/40	virus in extracellular medium		
0	979	206	0	401	372	0	37	616	
50	1084	435	6	519	124	46	78	35	151
150	380	85	0	198	97	97	0	34	195
200	11	9	0	0	0	0	0	9	22
300	0	0	0	0	0	0	0	0	14

3.4.4. SDS PAGE investigation of the MI and BHV-1 infected cell proteins labelling with ^{35}S -methionine and ^{32}P -orthophosphate.

Monolayers of MDBK cells were either MI or infected with BHV-1 at a m.o.i. of 20 p.f.u./cell. Following the virus absorption period, the monolayers were washed, overlaid with methionine-free, or phosphate-free medium (as appropriate) containing increasing concentration of drugs, and then labelled with either ^{35}S -methionine (at 3h PI), or ^{32}P - orthophosphate (at 4h PI). After overnight incubation, the cell monolayers were harvested in SDS PAGE extraction buffer, and the infected cell proteins examined by SDS PAGE. The effect of CCX treatment on the incorporation of ^{35}S -meth into MI and BHV-1 infected MDBK cell proteins is shown in Fig 38. Treatment with $\geq 75\mu\text{M}$ CCX resulted in a decrease of between 70% and 85% in the ^{35}S -methionine incorporation into MI or BHV-1 infected cells. There was little evidence to suggest that the drug acted selectively against BHV-1 and the data suggest that CCX treatment impairs protein synthesis per se.

Figure 39 shows the polypeptide profile of MI, or BHV-1 infected cell extracts from cultures treated with $0\mu\text{M}$, $12.5\mu\text{M}$, $25\mu\text{M}$, $50\mu\text{M}$, $75\mu\text{M}$, $100\mu\text{M}$, $125\mu\text{M}$, $150\mu\text{M}$, $200\mu\text{M}$, or $300\mu\text{M}$ CBX. In the MI cell extracts; bands of 53K, 51K, 40K, and 36K, were reduced in intensity when treated with CBX concentrations $>12.5\mu\text{M}$, while bands of 200K and 80K, were reduced in intensity by treatment with CBX concentrations $>75\mu\text{M}$. In addition, bands of, 79K, and 63K, increased in intensity in cultures treated with $>25\mu\text{M}$ CBX.

The following virus specific bands were identified in the extracts from drug-free infected cells; $> 200\text{K}$, 147K, 141K, 134K, 119K, 90K, 87K, 80K, 74K, 67K, 65K, 60K, 57K, 49K, 46K, 32K, and 28K. These bands probably correspond to the BHV-1 proteins

identified by Misra et al., (1981) as VPs; 1/2, 4, 5, 6, 7, 8, 9, 10/11, 12, 13, 14, 15, 16, 20, 21, 22, and 23.

The intensities of all viral bands were progressively reduced with increasing drug concentration, but the synthesis of some protein bands were less affected than others (VPs 4, 5, and 10/11, were detected at 300 μ M CBX, but VPs 1/2, 6, 7, 8, 12, 13, 14, 16, 20, 21, 22, and 23 were not detected with >100 μ M CBX).

Earlier observations, (Dargan, Bandephadhay, Field and Subak-Sharpe, unpublished data) suggested that phosphorylation of the BHV-1 VP8 protein might be impaired in the presence of CCX. Figure 40 shows the effect of CCX, tunicamycin, monensin, and H7 on the polypeptide profile of uninfected MDBK cells labelled in parallel with 35 S-methionine and 32 P-orthophosphate. H7 is a well-known protein kinase C (PKC) inhibitor (Ito et al., 1988) included in this experiment as a control. Cellular bands of 180K and 165K were reduced in intensity at 300 μ M CCX. A novel cellular band appeared at 120K in cultures treated with >100 μ M CCX. Bands of 86K and 67K were more intense in tunicamycin treated cells. In addition a 115K novel band was detected in tunicamycin treated cells. As a result of treatment with either >50 μ M CCX, or tunicamycin treatment, but not with monensin or H7 the phosphate labelling of a 156K band was impaired. A novel phosphoprotein band of 86K Mwt was detected in cultures treated with \geq 100 μ M CCX.

Figure 41 shows the parallel 35 S-methionine and 32 P-orthophosphate labelling of BHV-1 infected MDBK cell proteins. The following BHV-1 bands were identified in the extracts from drug-free infected cell cultures labelled with 35 S methionine; VPs 1/2, 4, 5, 6, 7, 8, 9, 10/11, 12, 13, 14, 15, 16, 20, 21. The intensities of the following viral bands were

virtually unaffected by treatment with 300 μ M CCX; 4, 5, 6, 10/11, 15, 16, 20, 21, while the intensities of 1/2, 7, 8, 9, 12, 13, 14, bands were reduced. Two bands of 66K and 55K were increased in intensity in infected cultures treated with $\geq 50\mu$ M CCX. A novel band of 38.5K appears as a result treatment with $\geq 25\mu$ M CCX, this band is not present in tunicamycin, monensin, or H7 treated cell extracts. The intensity of the VP8 band was unaffected with treatment up to 200 μ M CCX, although the intensity of this band was greatly reduced when treated with 300 μ M CCX. Phosphate labelling of VP8, however, was increased when treated with 25 μ M CCX, decreased when treated with $>50\mu$ M CCX, and was virtually undetectable at 200 μ M CCX. Phosphate labelling of a protein band at 85K was impaired in cultures treated with $>100\mu$ M CCX. Treatment with tunicamycin or H7 seemed to have little effect on the phosphorylation of VP8, but treatment with monensin impaired its phosphorylation. Treatment with tunicamycin reduced the intensities of 35 S-methionine labelled viral bands VP1/2 and VP14, and also resulted in induction of novel bands of 92K, and 67K, (the latter corresponds to a MI band). Phosphorylation of VP8 was not impaired in the presence of H7 indicating that, VP8 phosphorylation was not mediated through the action of protein kinase C. Recent data suggested that VP8 has protein kinase C activity and is capable of autophosphorylation (V. M., Misra personal communication). Whether phosphorylation of VP8 is essential for virus replication is not known, but the fact that CCX treatment impaired its phosphorylation under conditions where the protein was synthesised is of interest. It is also relevant, that the Golgi inhibitor monensin impaired the phosphorylation of VP8, but not synthesis of the VP8 protein itself (Fig 41 lanes 9 and 19).

As CCX also affects the Golgi apparatus, this implicates a Golgi function involved in phosphorylation of VP8.

In conclusion the results indicate that CBX treatment reduces protein synthesis per se, but some BHV-1 proteins were ^omore affected than the others, and that CCX treatment impaired the phosphorylation of VP8 at drug concentrations which did not affect the synthesis of the VP8 protein.



Figure 38.

The effect of CCX on incorporation of ³⁵S-methionine into MI or BHV-1 infected MDBK cell proteins.

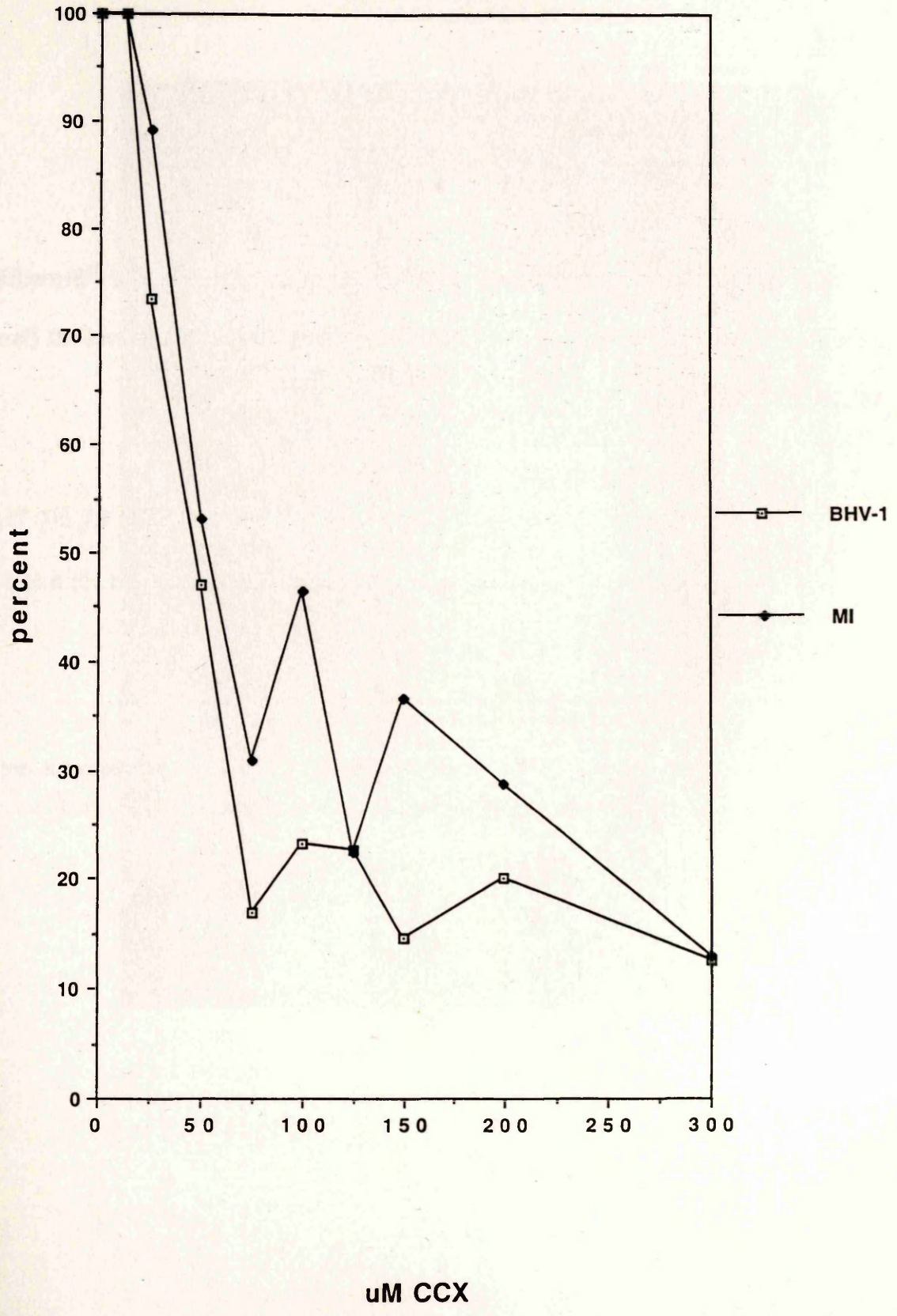


FIGURE 39.

Polypeptide profile obtained on a 10% SDS PAGE gel of the ^{35}S -methionine labelled proteins in the extracts from BHV-1 infected (lanes 1 to 10), and MI (lanes 11 to 20) of MDBK cells.

Cells were either drug-free (lanes 1 and 11), or treated with 12.5, 25, 50, 75, 100, 125, 150, 200, or 300 μM CBX (lanes 2 and 12; 3 and 13; 4 and 14; 5 and 15; 6 and 16; 7 and 17; 8 and 18; 9 and 19; 10 and 20 respectively).

Identified viral proteins (VPs) (Misra et al., 1981).

The apparent molecular weight of cellular proteins affected by CCX treatment are also shown.

- Bands decreasing in intensity with increasing drug concentration.
- Bands increasing in intensity with increasing drug concentration.

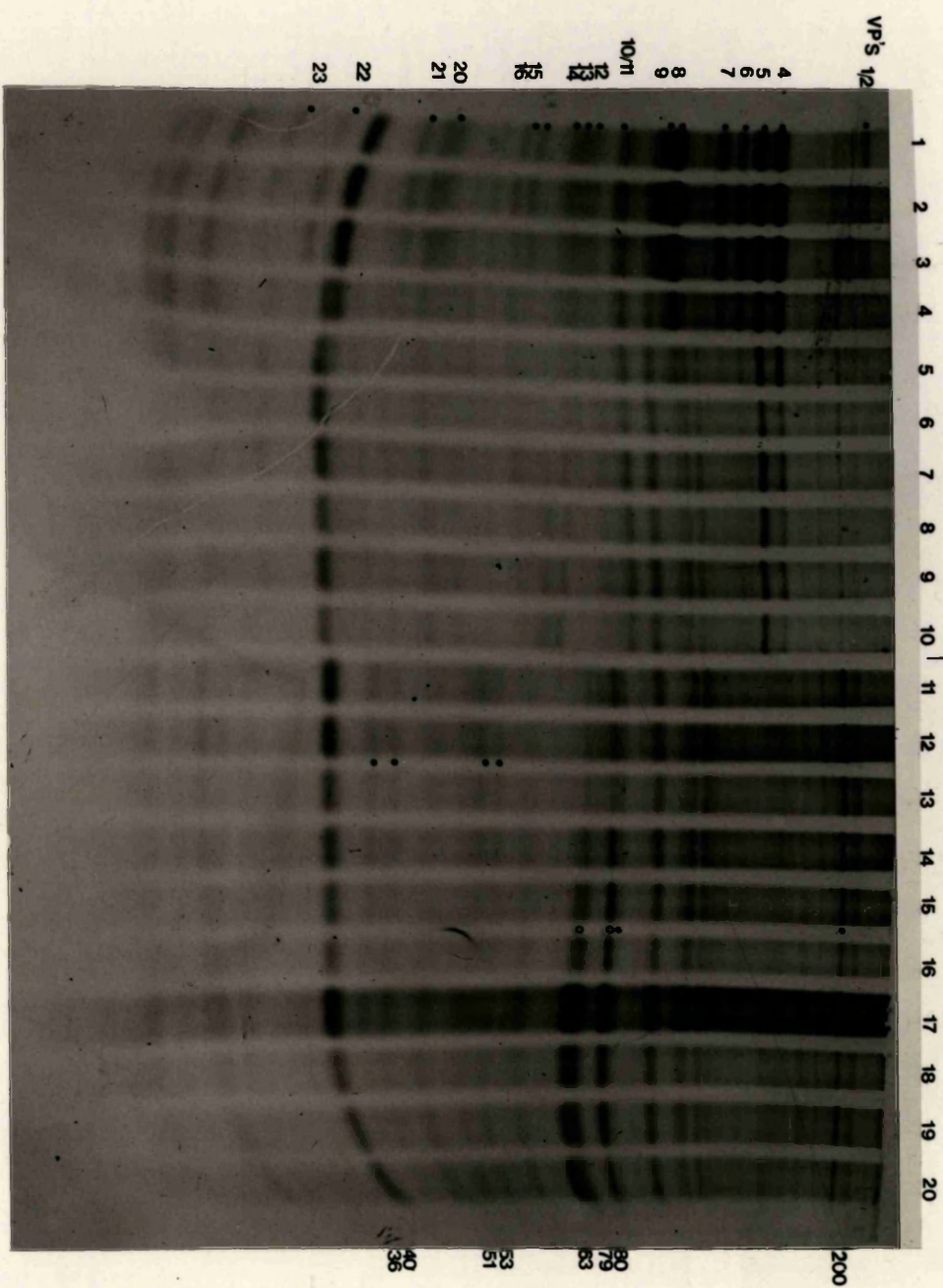


FIGURE 40.

Polypeptide profiles obtained on a 9% SDS PAGE gel of MI MDBK cell extracts from cultures labelled with either ^{35}S -methionine (lanes 1 to 10), or ^{32}P -orthophosphate (lanes 11 to 20).

Cells were either drug-free (lanes 1 and 11) or treated with 25, 50, 100, 150, 200, or 300 μM CCX (lanes 2 and 12; 3 and 13; 4 and 14; 5 and 15; 6 and 16; 7 and 17 respectively), 2 $\mu\text{g/ml}$ tunicamycin (lanes 8 and 18), 5 μM monensin (lanes 9 and 19), or 1 μM H7 (lanes 10 and 20).

- Bands increasing in intensity with increasing drug concentration.
- Bands decreasing in intensity with increasing drug concentration.
- Novel bands.

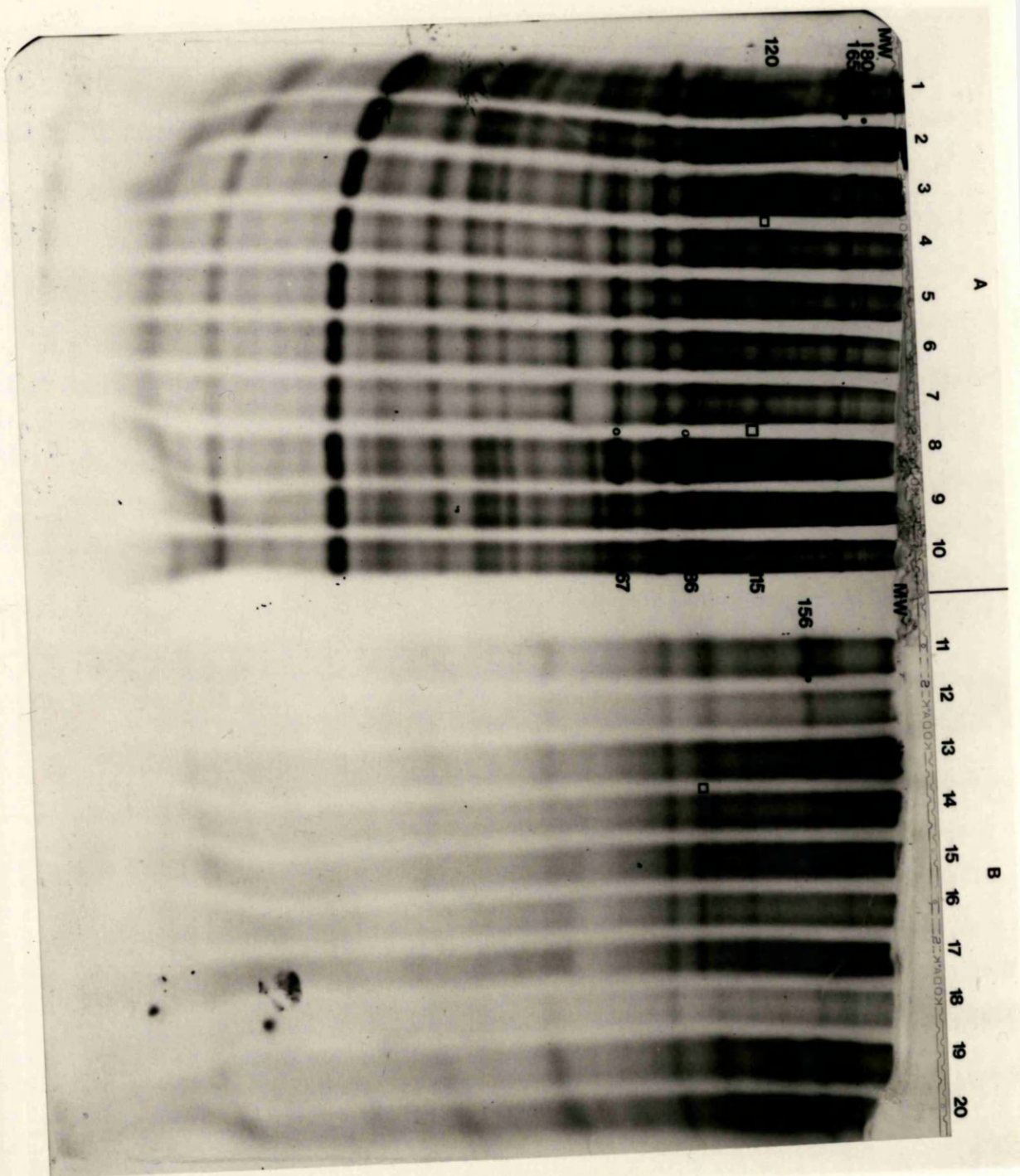


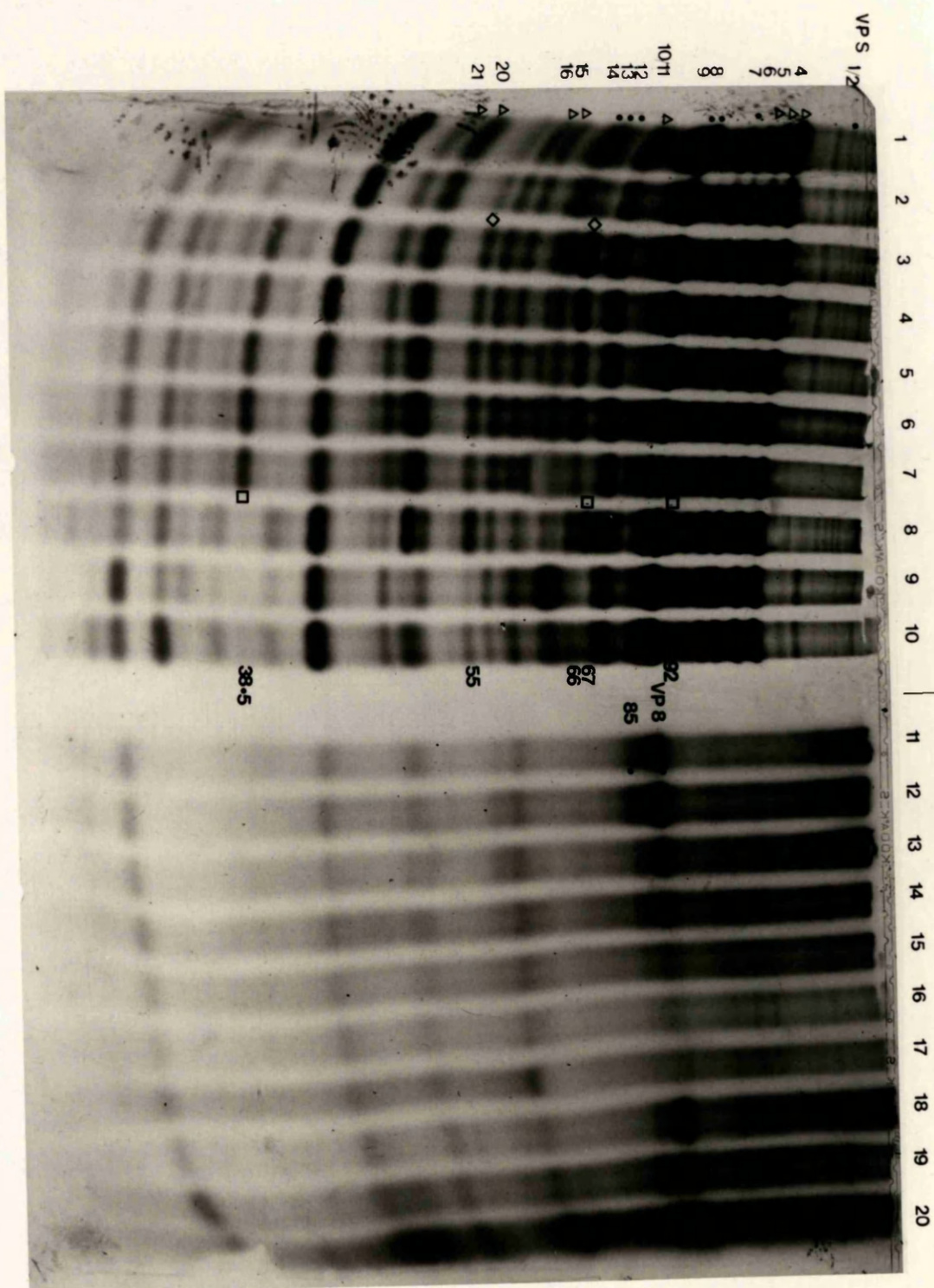
FIGURE 41.

Polypeptide profiles obtained on a 9% SDS PAGE gel of BHV-1 infected MDBK cell extracts from cultures labelled with either ^{35}S -methionine (lanes 1 to 10), or ^{32}P -phosphate (lanes 11 to 20).

Cells were either drug-free (lanes 1 and 11) or treated with 25, 50, 100, 150, 200, 300 μM CCX (lanes 2 and 12; 3 and 13; 4 and 14; 5 and 15; 6 and 16; 7 and 17 respectively), 2 $\mu\text{g/ml}$ tunicamycin (lanes 8 and 18), 5 μM monensin (lanes 9 and 19) and 1 μM H7 (lanes 10 and 20).

Identified viral proteins (VPs) (Misra *et al.*, 1981).

- Bands decreasing in intensity with increasing drug concentration.
- ◻ Novel bands
- △ Bands unaffected with increasing drug concentration.
- ◇ Bands increasing in intensity with increasing drug concentration.



4.1. CYTOTOXICITY TESTS.

The triterpenoid compounds; CCX, CBX, and GI, are hydrophobic and affect cell membrane functions, it was important therefore, to exclude cell death as a contributing factor in the antiviral activity. In order to study the antiviral activity of the drugs, cell lines resistant to the drugs had to be identified.

Dargatzis and Subak-Sharpe (1983), and Galt *et al* (1990) placed 23 different cell lines into one of three classes (resistant, intermediate, sensitive) based on tolerance to 48h treatment with up to 300µM CCX. The average cell viabilities obtained for resistant, intermediate, and sensitive classes of cell lines (Galt, *et al*, 1990) are shown in table 4.1.

CCX-sensitive cell lines had poor tolerance (12%) to 48h exposure with 100µM CCX; cell lines belonging to the intermediate class were only ~ 50% viable, when treated with 100µM CCX for 48h and viability of cell lines placed in the CCX-resistant class were in general hardly affected by treatment with CCX concentrations up to 300µM.

DISCUSSION

The viabilities of the CCFK and PeA cell lines employed in this study were little affected by treatment with up to 300µM CCX. Although treatment with 300µM CCX resulted in a significant (PeA) or slight (CCFK) loss of viability. Despite the loss of viability at 300µM CCX, the data obtained for the CCFK and PeA cell lines more closely approximated that obtained for cell lines classified by Galt *et al* (1990) as CCX-resistant, rather than intermediate or sensitive. It was, therefore, concluded that CCFK and PeA cells could be placed within the CCX-resistant group of cell lines.

4.1. CYTOTOXICITY TESTS.

The triterpenoid compounds; CCX, CBX, and GL are lipophilic and affect cell membrane functions, it was important therefore, to exclude cell death as a contributing factor in the antiviral activity. In order to study the antiviral activity of the drugs, cell lines resistant to the drugs had to be identified.

Dargan and Subak-Sharpe (1985), and Galt *et al* (1990) placed 23 different cell lines into one of three classes (resistant, intermediate, or sensitive) based on tolerance to CCX treatment (table 11). The IC₅₀'s obtained for CrFK and FeA cells were >300μM (24h) and >150μM (48h) for both cell lines (table 11). The RIs obtained for CrFK and FeA cells treated with IC₅₀ concentration of CCX at 24h were > 0.94 and > 0.84 respectively and after 48h treatment respectively. Thus the RIs obtained were greater than 0.43 and 0.67 after 48h treatment respectively. The average cell viabilities obtained for resistant, intermediate, and sensitive classes of cell lines (Galt, *et al.*, 1990) are shown in table 10. CCX-sensitive cell lines had poor tolerance (12%) to 48h exposure with 100μM CCX; cell lines belonging to the intermediate class were only ~ 50% viable, when treated with 100μM CCX for 48h and viability was rapidly reduced at higher drug concentrations; the sensitive (0.37 and 0.26 respectively) and intermediate (0.29 and 0.26 respectively) cell lines. It is, therefore, concluded that the CrFK and FeA cell lines should be placed at the higher end of the CCX-resistant cell grouping. The viabilities of cell lines placed in the CCX-resistant class were in general hardly affected by treatment with CCX concentrations up to 300μM.

4.2. ANTI-CCX ANTIBODY

The viabilities of the CrFK and FeA cell lines employed in this study were little affected by treatment with up to 200μM CCX, although treatment with 300μM CCX resulted in a significant (FeA) or sharp (CrFK) loss of viability. Despite the loss of viability at 300μM CCX, the data obtained for the CrFK and FeA cell lines more closely approximated that obtained for cell lines classified by Galt *et al* (1990) as CCX-resistant, rather than intermediate or sensitive. It was, therefore, concluded that CrFK and FeA cells could be placed within the CCX-resistant group of cell lines.

TABLE 10. Average percent cell viability determined at 48h after CCX treatment.

The data obtained by Dargan and Subak-Sharpe (1985) and Galt *et al* (1990) for CCX resistant cell lines revealed that, while cell viability remained high the growth rate of the culture was reduced. This was also found to be the case for CrFK and FeA cells treated with CCX and CBX (Figs; 7A, 7B, 12A, 12B). Galt *et al.*, (1990) have found that most of the cell lines that fell into the CCX-resistant class had IC50 concentrations (concentration of the drug inhibiting cellular growth by 50%) of > 300µM after 24h or 48h after drug treatment (table 11). The IC50's obtained for CrFK and FeA cells were >300µM (24h) and 150µM (48h) for both cell lines (table 11). The RI's obtained for CrFK and FeA cells treated with IC50 concentration of CCX at 24h were > 0.68, and > 0.84 respectively and 0.43 and 0.62 after 48h treatment respectively. Thus the RI's obtained were greater than the average RI values obtained at 24h and 48h with the IC50 concentration of drug for sensitive (0.37 and 0.26 respectively) and intermediate (0.29 and 0.26 respectively) cell lines. It is, therefore, concluded that the CrFK and FeA cell lines should be placed at the lower end of the CCX-resistant cell grouping.

4.2. ANTI-CCX ANTIBODY.

The cell-membrane binding properties of triterpenoid compounds suggest several possible targets for the drug e.g. plasma membrane, nuclear membrane, mitochondrial membrane, Golgi apparatus membrane, and/or various vesicular membranes. In order to investigate the intracellular location of CCX molecules, an attempt was made to generate an antibody against the drug.

As the CCX molecule has a molecular weight of only 668.8 daltons, it was considered to be too small to generate an immune response on its own- the molecule falls

TABLE 10. Average percent cell viability determined at 48h after CCX treatment.

CCX tolerance class, 48h	300µM	200µM	150µM	100µM
Sensitive*	ND	ND	2.6%	12.3%
Intermediate*	1%	16.6%	38.4%	56.4%
Resistant*	82%	82%	88%	90%
CrFK	9%	76.3%	80.2%	88.5%
FeA	58%	84%	95%	97%

ND. not determined.

*Data calculated from Galt et al., (1990).

* IC50- Defined as the concentration of the drug that inhibits cellular growth by 50% (Galt et al., 1990).

** RI-Replication Index is defined as multipliers indicating the increase in total number of cells at 24h or 48h relative to the total number of cells at day zero.

*** Data calculated from Galt et al., (1990).

Table 11. Average CCX IC50* and RI at IC50 of sensitive and intermediate cell lines in comparison to FeA and CrFK cells.**

Cell line	24h IC50	24h RI at IC50	48h IC50	48h RI at IC50
Sensitive***	88.6µM	0.37	48µM	0.26
Intermediate*	148µM	0.29	118µM	0.26
Resistant	>300µM	-	>300µM	-
CrFK	> 300µM	0.68	150 µM	0.43
FeA	> 300µM	0.84	150µM	0.62

* IC50- Defined as the concentration of the drug that inhibits cellular growth by 50% (Galt et al., 1990).

** RI-Replication index is defined as multipliers indicating the increase in total number of cells at 24h or 48h relative to the total number of cells at day zero.

*** Data calculated from Galt et al., (1990).

into the class of compounds known as haptens. To facilitate the generation of an immune response to CCX, the drug was conjugated-via a COONa group to a hydroxyl group on a BSA protein molecule.

Despite the apparent detection of a specific anti-CCX antibody by an ELISA test, subsequent immunofluorescence experiments to detect CCX failed to exhibit any specific reaction (data not shown). In a time-course experiment, in which control and drug-treated cells were fixed at various times after drug addition, "CCX-immune sera" gave a punctate nuclear fluorescence in both drug-treated and control cells even at time zero, (titrating out at 1/400), indicating that the fluorescence observed was non-specific (data not shown). The number of fluorescent foci, however appeared to be greater in CCX-treated cells. The pre-immune sera gave cytoplasmic and perinuclear fluorescence, (titrating at 1/3200) in drug-treated, and untreated cells (data not shown). Again the fluorescence was observed from the time of drug-addition suggesting that the fluorescence was non-specific. The pre-immune rabbit serum bound non-specifically to CCX-treated MDBK cells, and could not be removed by washing. It was possible that this non-specific reaction masked the fluorescence that might have come from a specific reaction between CCX and the anti-CCX antibody. At the present time, there seems to be no rational explanation for these confusing, but consistent observations obtained with different cell lines (4 experiments with MDBK cells, and two each with CrFK, and FeA cells).

In order to investigate whether the effect of CCX on Na^+/K^+ ATPase was relevant to the effect of the drug on the Golgi apparatus, ouabain (a compound which specifically inhibits Na^+/K^+ ATPase activity by binding to the α subunit of the molecule) was used in the Golgi immunofluorescence experiments. While p58-associated Golgi fluorescence was

4.3. THE EFFECT OF CCX ON THE GOLGI APPARATUS.

Previous studies (Dargan and Subak-Sharpe 1986, and 1986a; Galt *et al.*, 1990), have provided some evidence to suggest that the Golgi apparatus might be a target for triterpenoid compounds.

The Golgi apparatus consists of a series of flattened, disk-shaped, membrane bound compartments arranged in three distinct regions: the cis, medial, and trans Golgi compartments. Post-Golgi transport vesicles (the trans-Golgi network) are involved in intracellular transport of proteins, following their processing within the Golgi apparatus. (Ktistakis, N., *et al.*, 1991).

Individual Golgi compartments have been associated with particular processing events, and it is generally accepted that the glycosylases involved in building long sugar side-chains on glycoproteins are located in the trans-Golgi compartment

CCX treatment resulted in; a reduction in perinuclear (trans-Golgi; anti-p58 protein) fluorescence and the appearance of punctate intranuclear fluorescence (particularly in MDBK cells). These observations can be explained either by the disaggregation of the trans-Golgi compartment, or even the entire Golgi apparatus, and/or the redistribution of p58 protein to the nucleus of the drug-treated cells.

In order to investigate whether the effect of CCX on Na^+/K^+ ATPase was relevant to the effect of the drug on the Golgi apparatus, ouabain (a compound which specifically inhibits Na^+/K^+ ATPase activity by binding to the α subunit of the molecule) was used in the Golgi immunofluorescence experiments. While p58-associated Golgi fluorescence was

abolished in MDBK cells treated with CCX, the Golgi apparatus was labelled in cells treated first with ouabain and 1h later with CCX. Thus, CCX-induced up-regulation of the Na^+/K^+ ATPase was correlated with the perturbation of the trans-Golgi compartment and redistribution of p58 molecules.

It was postulated that, if the trans-Golgi compartment of MDBK cells was normally an acidic compartment, and the trans-Golgi membrane contained Na^+/K^+ ATPase molecules, it could be predicted that CCX-induced up-regulation of the Na^+/K^+ ATPase activity would result in neutralisation of the acidic environment of the cisternae (Na^+ ions would be pumped into the cisternae, while H^+ ions would be removed). To test this hypothesis the acidic compartments of the Golgi apparatus in MDBK CCX-treated cells were visualised using the DAMP/anti-DAMP system of Anderson *et al.* (1989). The results (section 3.6) confirmed that the trans-Golgi compartment of MDBK cells was acidic. In the presence of CCX, the acidic trans-Golgi compartment of MDBK cells was either neutralised, destroyed, or redistributed, in the form of numerous cytoplasmic vesicles. The effect of CCX-treatment on the other acidic compartments of the cells; e.g. endocytic vesicles, lysosomes (Anderson *et al.*, 1984), and post-Golgi vesicles (Fishkes *et al.* 1982) has not been investigated.

4.4. A MODEL TO EXPLAIN THE EFFECT OF CCX ON GOLGI APPARATUS.

A model which can account for the observed effects of triterpenoid compounds on the Golgi apparatus is shown in Figure (42). The model is based on the processing of

receptors and ligands in early endosomes and the effect of Na^+/K^+ ATPase on this process. Figure 42-(A) shows the normal intracellular route of processing of ligands and receptors. Receptor molecules are displayed on the plasma membrane (A-1). When a ligand binds to the receptor, it is internalised by receptor mediated endocytosis (A-2). After invagination, and pinching-off an early endosome is formed (A-3). Endosomes exist as two distinct sub-population (early, A-3 and A-4 and late, A-8) (Schmid, *et al.*, 1988). Early and late endosomes are functionally distinct. Early endosomes are CURL compartments (compartment of uncoupling of receptor and ligands) (A-4) (Anderson, *et al.* 1984). Late endosomes (A-8), on the other hand, are involved in delivering the ligand to lysosomes (A-9) (Schmid, *et al.*, 1988). Endosomes maintain an acidic environment by the action of an ATP dependent proton pump located on the membrane of endocytic vesicle (A-3), (Darrel, *et al.*, 1983). Upon acidification of the early endosome, the ligand dissociates from its receptors (A-4), a portion of the early endosome membrane containing the receptor then buds off, and is transported to the trans-Golgi for recycling to the plasma membrane (A-5)(A-6)(A-7). The early endosome then matures into the late endosome form (A-8), and the ligand is transported to the lysosome, in which it is degraded (A-9) (Brown, *et al.* 1983).

Figure 42 B shows the effect of Na^+/K^+ ATPase on the processing of the ligand and the receptor. Two classes of early endosomes, which acidify at different rates have been reported (Fuchs *et al.*, 1989). Occasionally Na^+/K^+ ATPase molecules are included during receptor-mediated endocytosis (B-2). Since, the action of the early endosome proton pump is under electrogenic control, [i.e. the more positive charge (H^+ or Na^+) inside the vacuole the harder it is to pump in more protons (H^+)], those early endosomes which have incorporated $\text{Na}^+/\text{K}^+/\text{ATPase}$ in their membrane, acidify at a slower rate (B-3) (B-3a)

(Fuchs, *et al.*, 1989). Eventually the pH of the endosome falls sufficiently to allow uncoupling of the ligand and receptor (B-4), with subsequent recycling of the receptor and Na^+/K^+ ATPase to the plasma membrane via the trans-Golgi system (B-5)(B-6)(B-7) (Fuchs *et al.*, 1989). The finding that, Na^+/K^+ ATPase impairs the acidification of some early endosomes was further supported by the observation that ouabain treatment resulted in a higher proportion of low pH endosomes (Cain, *et al.*, 1989).

Figure 42 C, shows the effect of CCX treatment on the processing of ligand and the receptor, when Na^+/K^+ ATPase is present. Triterpenoid compounds inhibit PKC-activity in *in vitro* assays (Ito *et al.*, 1988). PKC is thought to be responsible for regulation of Na^+/K^+ ATPase- activity via phosphorylation of the α -subunit of the pump. Phosphorylation inhibits the activity of the pump (Middelton *et al.*, 1993). It can be expected, therefore that in CCX-treated cells, those endosomes which have incorporated sodium pump molecules accumulate sodium ions rapidly (C-3)(C-3a). The competition between the proton pump and the Na^+/K^+ ATPase may seem to be weighted in favour of the latter, not only because the proton pump is electrogenic, but also, because the pump accepts H^+ in place of K^+ and consequently removes 3H^+ from the endosome, while pumping in 2 Na^+ ions (B-3) and (B-3a). If the pH drop is insufficient for the recycling of receptor or Na^+/K^+ ATPase molecules, the sodium pump will remain embedded in the endosomal membrane. The large influx of osmotically active Na^+ ions into the endosome will result in an inflow of H_2O into the endosome leading to swelling and formation of a large single-compartmented vacuole (C-3a) [such large single-compartmented macro-vacuoles have been observed in the cytoplasm of CCX-treated BHK and flow 2002 cells (Dargan and Subak-Sharpe, unpublished observations)]. If the proton pump successfully lowers the pH

of the endosome, it is likely that the $\text{Na}^+/\text{K}^+\text{ATPase}$ will be recycled with the receptor via the trans-cisternae of the Golgi apparatus (C-5). Those Na^+/K^+ ATPase molecules which are transported to the Golgi apparatus might then set up the same Na^+/H^+ competition with Golgi-resident proton pumps (C-5) (Glickman, *et al.* 1983), leading to neutralisation of the acidic environment of the trans-Golgi compartment (as shown in the Figure 18). An increase in Na^+ ion concentration in the trans-Golgi cisternae could result in influx of water into this compartment (C-10), leading to perturbation of Golgi functions. Large multi-compartmented vacuoles in CCX-treated cells (thought to represent disorganised Golgi apparatus) have been observed in BHK and Flow 2002 by Dargan and Subak-Sharpe (unpublished observations). Influx of water might result in the complete destruction of the trans-Golgi compartment, its disintegration into many smaller vesicles, and/or its gross disfiguration and swelling into unified multicompartmented vacuoles (C-15) (as shown in figure 17).

The model might also explain the earlier observation^{by} Galt *et al.*, (1990) that in general fibroblast cell lines are more sensitive to the cytotoxic effect of triterpenoid drugs, than are epithelial cell lines. Anderson *et al.* (1985) have shown that, in general, fibroblast cell lines have an acidic trans Golgi compartment, while that of epithelial cell lines is neutral. Both cell lines, however, have an acidic post-Golgi network.

FIGURE 42A. THE MODEL EXPLAINING THE EFFECT OF CCX ON GOLGI APPARATUS.

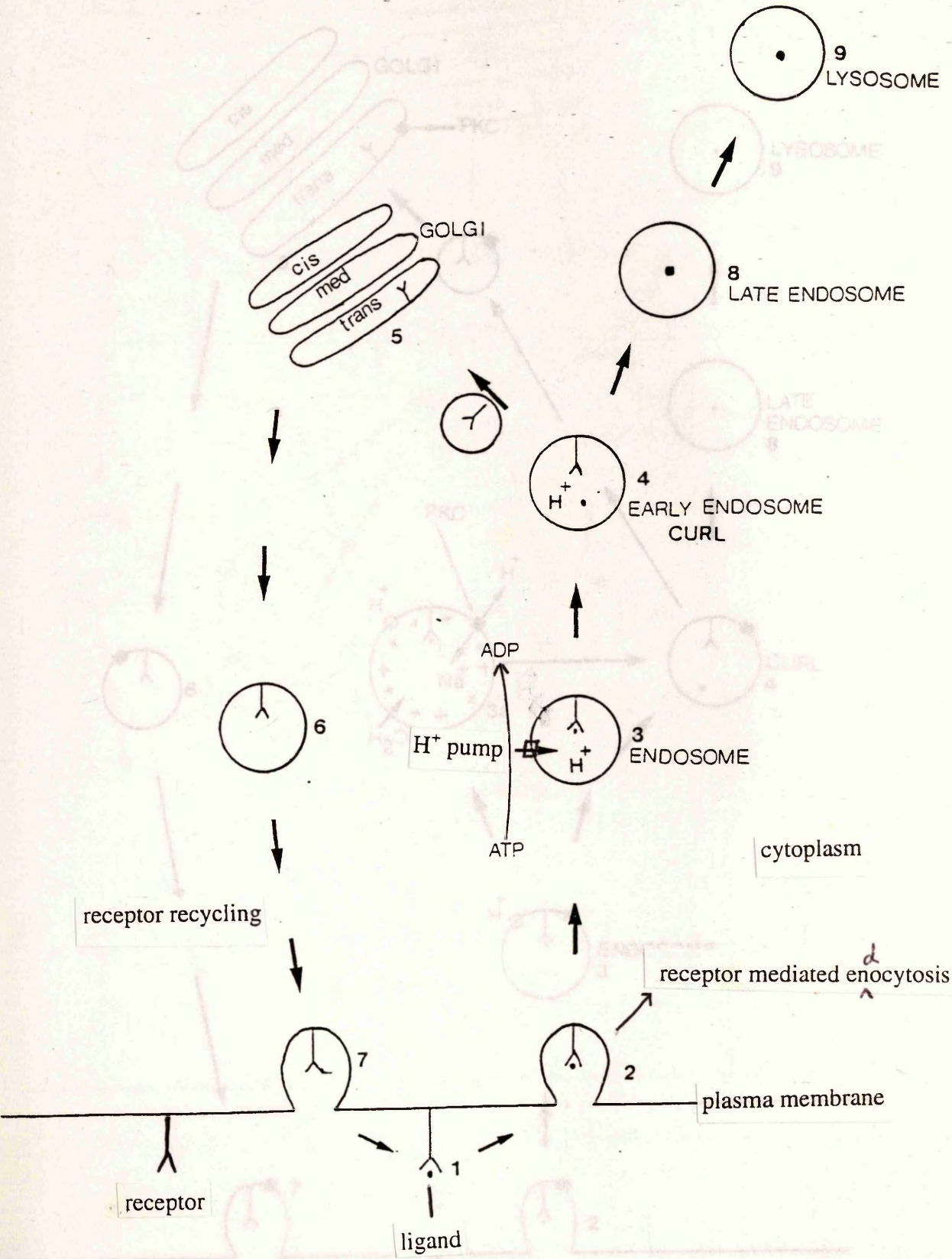


FIGURE 42B. THE MODEL EXPLAINING THE EFFECT OF CCX ON GOLGI APPARATUS.

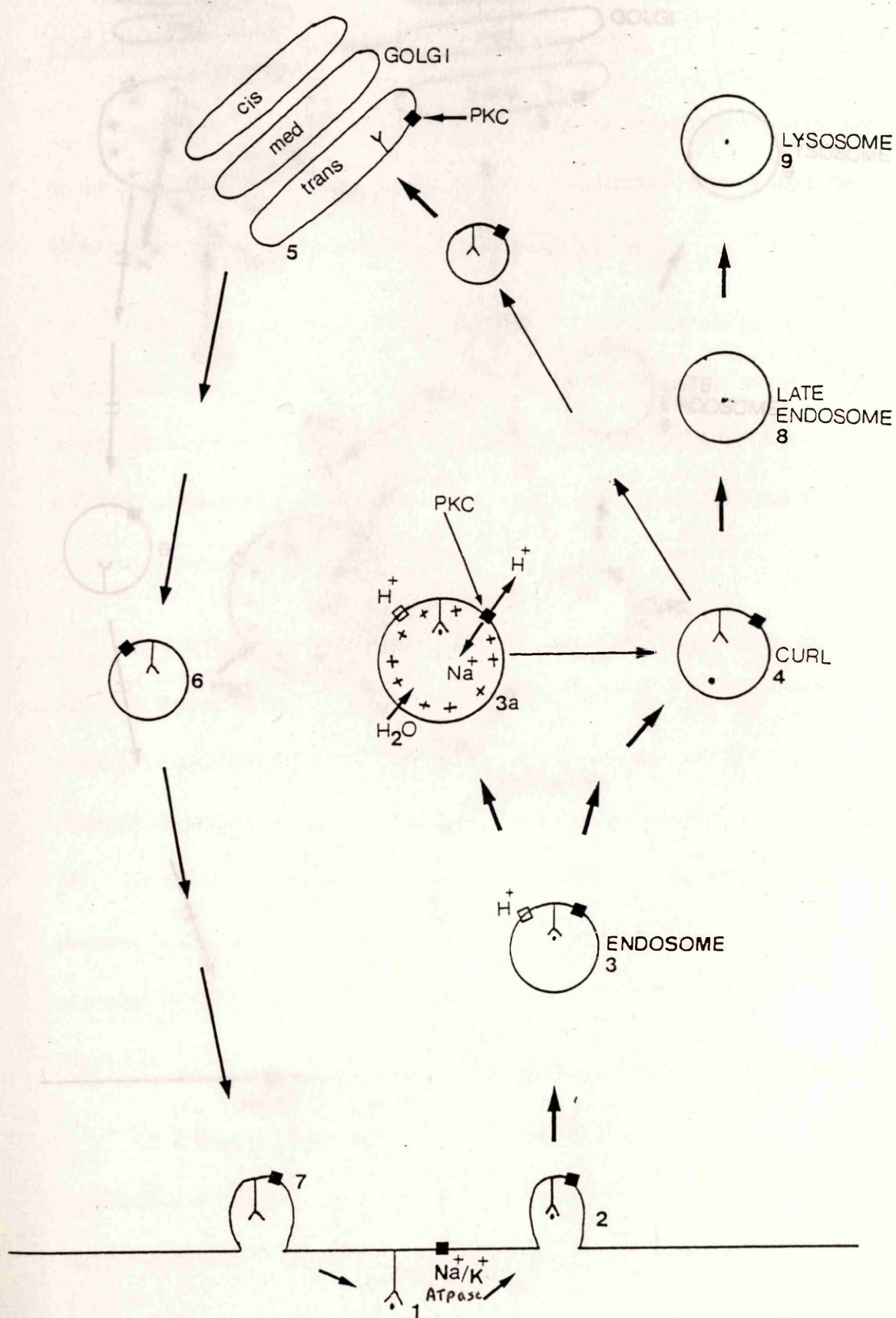
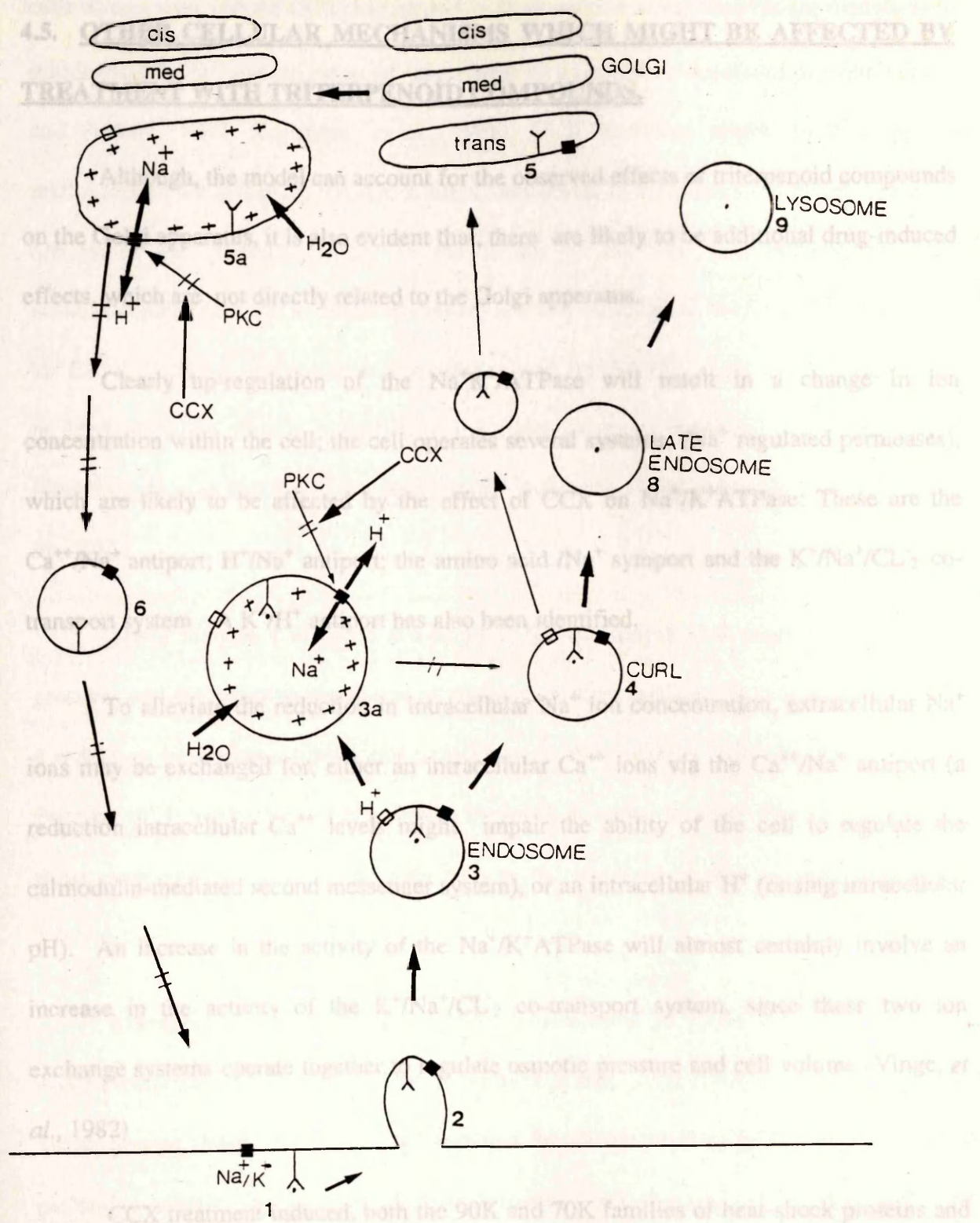


FIGURE 42C. THE MODEL EXPLAINING THE EFFECT OF CCX ON GOLGI APPARATUS.



4.5. OTHER CELLULAR MECHANISMS WHICH MIGHT BE AFFECTED BY TREATMENT WITH TRITERPENOID COMPOUNDS.

Although, the model can account for the observed effects of triterpenoid compounds on the Golgi apparatus, it is also evident that, there are likely to be additional drug-induced effects, which are not directly related to the Golgi apparatus.

Clearly up-regulation of the $\text{Na}^+\text{K}^+/\text{ATPase}$ will result in a change in ion concentration within the cell; the cell operates several systems, (Na^+ regulated permeases), which are likely to be affected by the effect of CCX on $\text{Na}^+/\text{K}^+/\text{ATPase}$: These are the $\text{Ca}^{++}/\text{Na}^+$ antiport; H^+/Na^+ antiport; the amino acid $/\text{Na}^+$ symport and the $\text{K}^+/\text{Na}^+/\text{CL}_2^-$ co-transport system. A K^+/H^+ antiport has also been identified.

To alleviate the reduction in intracellular Na^+ ion concentration, extracellular Na^+ ions may be exchanged for, either an intracellular Ca^{++} ions via the $\text{Ca}^{++}/\text{Na}^+$ antiport (a reduction intracellular Ca^{++} levels might impair the ability of the cell to regulate the calmodulin-mediated second messenger system), or an intracellular H^+ (raising intracellular pH). An increase in the activity of the $\text{Na}^+/\text{K}^+/\text{ATPase}$ will almost certainly involve an increase in the activity of the $\text{K}^+/\text{Na}^+/\text{CL}_2^-$ co-transport system, since these two ion exchange systems operate together to regulate osmotic pressure and cell volume (Vinge, *et al.*, 1982)

CCX treatment induced, both the 90K and 70K families of heat-shock proteins and the families of 94K and 78K, glucose-regulated of proteins, in all cell lines tested (Dargan and Subak-Sharpe, 1986 and Galt, PhD thesis 1989) (70K protein was also present in CrFK

cells treated with 100 μ M CCX; Figure 34A) . Such cellular stress proteins are thought to be responsible for the solubilization of aggregates of unfolded or denatured proteins (Sorger and Pelham, 1987; Kozutsumi, *et al.*, 1988). CCX treatment might result in protein misfolding or denaturation, through a direct interaction between the drug molecules in the membrane and proteins present in the lumen of the ER. Alternatively, excessive accumulation of proteins in the ER cisternae, due to a blockage in the flow of proteins from the ER to the Golgi, then to other intracellular sites (model 42), might result in misfolding or denaturation of proteins in the ER or the Golgi apparatus contents. In this regard it is interesting to note that grp94 has been located within the Golgi apparatus itself (Welch, *et al.*, 1983).

Stress induces a vigorous activation of the transcription of genes encoding stress proteins, and the selective translation of the stress protein mRNA. This is accompanied by a reduction in the transcription and translation of other cellular mRNAs (Ashburner and Bonner, 1979; Schlesinger *et al.*, 1982). Therefore it seems possible that induction of the stress response by CCX, might contribute to the impaired growth of drug-treated cell cultures.

Triterpenoid compounds have been demonstrated to affect the integrity of the plasma membrane resulting in leakage of proteins at high drug concentrations (Dargan and Subak-Sharpe 1986). GL was found to affect membrane fluidity by decreasing the lipid packaging density of cell membranes (Reardon and Audus, 1993). Although, the cytotoxicity studies shown here for FeA and CrFK cells have indicated that such effects (if

induced by the concentrations used here) are non-lethal, the effects are likely to impair cell culture growth.

4.6. ANTIVIRAL ACTIVITY AGAINST FELV-A.

Drug-dose response experiments with FeLV-A revealed that, a 100-150 fold reduction in infectious virus yield was obtained with CCX, CBX, or GL treatments. With all of the drugs, the infectivity of the CR virus yield decreased at a faster rate than that remaining cell-associated. This decrease in CR virus infectivity was not entirely due to a virucidal effect of the drug, suggesting that the drugs must impair the release of infectious virus from the cell surface. This implies that virus maturation can occur while virus particles remain attached to the cell surface. The kinetics of the FeLV-A dose-response curves obtained were similar for each drug and characterised by a triphasic curve. This suggests that the triterpenoid compounds have at least two separate targets. The first is sensitive to low drug concentration and provides either an essential function, which is only partially impaired by drug treatment or a function which enhances, but is not strictly essential for virus replication. The second target is sensitive to high drug concentrations and affects functions which appear to be essential for virus replication. At the relatively high drug concentrations required to inhibit the second of these targets, the window between antiviral activity and cytotoxicity is very narrow.

Comparison of the CCX dose-response curves obtained for FeLV-A with those obtained by Galt *et al.*, (1990), reveals that FeLV-A can be placed within the CCX^s-2 class along with Bunyaviruses, Adenoviruses, Reoviruses, and Polioviruses. The dose response curves for each of these viruses displayed a sharp initial decrease in infectious virus yield at

low drug concentration, followed by a plateau in the curve. Furthermore, the infectious yield reduction (~ 100-150 fold) for each virus was also similar. CCX treatment of FeLV-A infected cells did not seem to impair viral protein synthesis, although the protein composition of the virus progeny produced in the presence of the drug varied from that of the control (gp70 was decreased, while p15 was unaffected), suggesting some effect at the level of virus assembly. Interestingly, the SDS PAGE gels provide no evidence for impaired virus glycoprotein processing in CCX-treated FeA cell cultures. Unfortunately, the anti-p58 (trans-Golgi specific) antibody failed to work with the FeA cell line and so it was not possible to investigate the effect of CCX on Golgi apparatus labelling.

4.6.1. PROPOSED ANTIVIRAL MECHANISM AGAINST FELV-A.

One possible explanation for the antiviral activity against FeLV-A might be that FeA cells do not have an acidic trans-Golgi compartment, and therefore, glycoprotein processing was not significantly affected. However, it is likely that post-Golgi transport vesicles are acidic compartments (Anderson et al., 1985), and it might be that transport of glycoproteins which are contained within those post-Golgi vesicles, also recycling Na^+/K^+ ATPase molecules is inhibited. Changes in the H^+ concentration in such vacuoles might inhibit transport of the vacuole and result in a decrease in the amount of gp70 reaching the plasma membrane. The 100-150 fold reduction in infectious virus yield then might be due to a lack of sufficient glycoproteins in the plasma membrane to allow particle assembly at normal rates. Presence of CCX molecules within the plasma membrane, or

incorporated into the virus envelope, might impair particle release, and/or maturation of the virions.

4.7. ANTIVIRAL ACTIVITY AGAINST FIV.

Because FIV generally grows to a low titre, it was not possible to perform high MOI dose-response curve experiments with this virus, and so antiviral activity was demonstrated by plaque reduction assay and determination of ED50 concentration (75µM for CCX and 101 for CBX). Although, the triterpenoid drugs exhibited virucidal activity against FIV at the concentrations used in antiviral experiments, this was of low magnitude (at best 10 fold reduction in infectious virus yield after 24h incubation at 37°C).

SDS PAGE analysis of the protein composition of FIV progeny released from cells treated with 100µM CCX, showed that the p55 protein was overabundant. While p24 and p17 were present in reduced amounts, suggesting impaired cleavage of p55 into the p24 and p17 products. As this cleavage event is thought only to occur after particle assembly; during or after, particle release from the cell it seems likely that CCX treatment impairs FIV maturation.

The mature form of the FIV surface glycoprotein (gp120) was detected in extracts from drug-free cultures, and cultures treated with 25µM CCX, but not 100µM CCX. Suggesting either, that the glycosylation process was inhibited by the drug or that the precursor protein was not made. The lack of a FIV glycoprotein specific antibody meant that immature or partially glycosylated ³⁵S-labelled FIV glycoproteins could not be

unambiguously identified. With regard to the FIV transmembrane protein gp41; this protein was not detected by ^{14}C -glucosamine labelling, but does label with ^{35}S -methionine/cysteine and increased in amount in cells treated with 100 μM CCX. Interestingly, a ^{35}S -meth/cys labelled band of about 160K (probably the FIV glycoprotein precursor gp160) was observed to increase in amounts in cells treated with 100 μM CCX. These results are explainable in terms of drug induced impairment of either post-Golgi transport of the glycoprotein to the plasma membrane, or impairment of the assembly and/or release of progeny virus.

4.7.1. PROPOSED ANTIVIRAL MECHANISM AGAINST FIV.

A model to explain the anti-FIV effect of CCX is explained based upon the effect of CCX on the trans-Golgi compartment. Both glycosylation and the passage of glycoproteins through the Golgi system is inhibited. The accumulation of the precursor glycoprotein gp160 suggests that cleavage of gp160 into gp120 and gp41 is impaired. This could be due to slow ER to Golgi transport, resulting in accumulation of gp160 within the ER. As the cleavage of gp160 into gp120 and gp41 did not seem to be totally abolished, it seems possible that this cleavage event might occur in an earlier (cis or medial) Golgi compartment. Impaired transport of virus glycoproteins from trans-Golgi compartment to the plasma membrane via the post-Golgi vesicles, would result in reduced expression of viral glycoproteins (probably incompletely processed) on the cell surface. This might limit the number of virus particles that can be assembled (and also account for the observed accumulation of gp160, gp41, and p55 proteins within CCX-treated cells). It should be noted, however, that in general there is a fairly rapid turnover of Golgi apparatus (turnover

time ~ 20-30 minutes), in uninfected cells and so the drug may not completely abolish either glycosylation or post-Golgi transport of virus glycoproteins.

Budding and release of the virus from the plasma membrane may also be impaired; whether this is due to the presence of CCX molecules at the site of virus budding, incorporation of CCX into the envelope of the virion, or the lack of sugar-residues on the glycoproteins is not known. Post-release maturation of the virus particle does seem to be impaired, because the cell-free particles produced in the presence of CCX, either process p55 into p24 and p17 at a slower rate, or there is a population of virus particles in which p55 processing is completely inhibited. Whether the impaired cleavage of p55 into p24 and p17 resulted from an effect of the drug on the FIV protease activity, or whether CCX drug molecules embedded in the virus envelope directly or indirectly interfere with the protease activity is not known.

4.8. ANTIVIRAL ACTIVITY AGAINST BHV-1.

In contrast to the relatively weak antiviral activity of triterpenoid compounds on the replication of FeLV-A, the anti BHV-1 activity was very potent (for BHV-1 treatment with 150µM CCX, gave 3435 fold reduction in CR infectivity and 200 fold reduction in CA infectivity, compared to 155 fold reduction in CR infectivity and 190 fold reduction in CA infectivity for FeLV-A).

The magnitude of the anti-BHV-1 activity and the kinetics of the drug-dose response curve obtained in the experiments reported here, confirms the placement of BHV-

1 in the CCX sensitive class of viruses, described by Galt *et al.* (1990). Interestingly, BHV-1 CR infectivity decreased at a faster rate than CA infectivity, this observation was also made for HSV-1 (Dargan and Subak-Sharpe, 1986) and for FeLV-A. Virucidal activity of the drugs can account for part of the loss of infectivity of retroviruses (FIV and FeLV-A), but in the case of BHV-1, where virucidal activity could only account for ~ 4 fold loss of infectivity (at 300 μ M CCX), additional factors must be responsible for virtually all of the observed loss in infectivity. The data thus implicate, impaired release of BHV-1 progeny particles and /or the release of non-infectious progeny virus.

A quantitative electron microscope investigation, in which the numbers of BHV-1 particles, or virus-related structures, present in cell sections of CCX-treated cultures were counted, revealed that with increasing drug concentrations: (1) The percentage of cells containing BHV-1 particles decreased. (2) Fewer virus particles were assembled in each infected cell. (3) Transport of DNA containing virus particles from the nucleus to the cytoplasm was impaired. (4) The percent extracellular virus particles was decreased. Thus, the data show that CCX-treatment impaired both virus particle assembly and maturation.

Interestingly, the data also show that treatment with up to 150 μ M CCX had little, or no effect, on BHV-1 DNA packaging since the percentage of DNA-cored particles remained 60%-80% of the total virus particles in the cell. This observation is in accord with the findings of Dargan and Subak-Sharpe (1986a) that HSV-1 DNA synthesis was not impaired by CCX or CBX treatment.

SDS PAGE analysis of BHV-1 infected cell extracts obtained from cultures grown in the presence or absence of triterpenoid compounds, showed an overall decrease in the

amount of virus proteins synthesised (~ 90% decrease at 300 μ M CCX Fig 38), however, not all proteins were affected to the same extent (Fig 41). While CCX treatment of MDBK cells resulted in a general suppression of protein synthesis per se (to about 10% of the control level), the finding that all BHV-1 proteins were not affected equally deserves further study. Although the effect of CCX on protein synthesis per se must contribute to the reduction in the number of virus particles assembled, it can not by itself account for the greater part of the observed anti-BHV-1 activity of the drugs.

300 μ M CCX treatment specifically impaired the phosphorylation of the BHV-1 VP8 protein, a tegument protein with protein kinase activity (Carpenter and Misra, 1991). It was thought possible that, if VP8 was phosphorylated by PKC, then CCX-induced inhibition of PKC activity (Ito *et al.*, 1988) could explain this observation. Experiments employing the PKC inhibitor H7, however, showed that VP8 phosphorylation was not inhibited, demonstrating that PKC was not involved in VP8 phosphorylation. More recent data (Misra personal communication) has suggested that VP8 is capable of autophosphorylation. The mechanism by which CCX inhibits autophosphorylation of VP8, is currently under investigation by Misra and co-workers. It is not yet known, whether VP8 is an essential virus protein or whether its phosphorylation is required for its activity.

4.8.1. PROPOSED ANTIVIRAL MECHANISM AGAINST BHV-1.

Both BHV-1 and HSV-1 are α -herpesviruses. The anti-HSV-1 activity of CCX has been studied in detail by Dargan and Subak-Sharpe (1985, 1986, and 1986a). The anti-

HSV-1 activity was comprised of 3 components; 1. A virucidal activity; 2. A decrease in the number of viral proteins synthesised; 3. An increase in particle/p.f.u. ratio of the progeny virus made in the presence of the drug.

4.9. FINAL CONCLUSIONS

The virucidal activity of triterpenoid compounds against BHV-1 could account for as much as a 4 fold reduction in BHV-1 infectivity. This is of a similar magnitude of the virucidal activity obtained against HSV-1. CCX treatment of uninfected or HSV-1 infected BHK or Flow 2002 cells resulted in a decrease in the protein synthesis per se ($\geq 90\%$ at $300\mu\text{M}$), and a similar reduction in protein synthesis was found for uninfected or BHV-1 infected MDBK cells. A reduction of up to 90% in protein synthesis in drug-treated infected cells must play a role in decreasing the numbers of virus particles that can be assembled. Although, electron microscopy studies showed that, CCX had no effect on the envelopment of BHV-1. maturation of the progeny virus particles made in the presence of the drugs was impaired. Eleven, of forty, identified BHV-1 proteins labelled with glucosamine (Misra *et al.*, 1981). Although the requirement for virus infectivity of addition of sugars to these glycoproteins has yet to be studied, it is clear, from immunofluorescence experiments that CCX treatment resulted in disruption of the Golgi apparatus of MDBK cells. It would seem reasonable to suspect that in the MDBK cell line used, impaired glycosylation of BHV-1 glycoproteins would also play a role in the antiviral activity against BHV-1. Incorporation of CCX molecules into the various cell membranes might also have a role in the antiviral effect. Although, the drugs did not appear to inhibit the envelopment of BHV-1, the infectious titres decreased in the CR fraction at a faster rate than the CA titre, suggesting that the drugs impair the release of the infectious virus particles into the growth medium. An alternative explanation is that virus particles

produced and released in the medium in the presence of CCX are less stable than those produced under drug-free conditions.

4.9. FINAL CONCLUSIONS

A full and complete comparison of the antiviral effects of triterpenoid compounds on the replication of FIV and FeLV-A (retroviruses) and the BHV-1 (herpesvirus) was hindered, because not all experiments were completed for each virus; in part this was due to technical difficulties with particular virus/cell systems, and in part due to a lack of time to follow up these studies. Nevertheless, a list of common features can be given; 1. The drugs have broad range antiviral activity; 2. Treatment with the drugs resulted in a faster decrease in CR than CA infectivity; 3. The faster decrease in CR infectivity was not completely explained by the virucidal effect of the drugs; 4. The drugs impaired the release of virus particles from the cell.

The following scheme describes current thinking on the mechanism of anti-retrovirus action of the triterpenoid drugs.

Drug molecules bind to the plasma membrane due to their hydrophobic nature, and become embedded in the membrane; with time the drug molecules become distributed throughout the cell and are probably membrane bound (Dargan and Subak-Sharpe, unpublished observations). The drugs impair PKC activity and so upset those cellular processes normally regulated by this ubiquitous enzyme (it is also possible that drug molecules also interfere with other cell membrane signalling systems). Impaired PKC

activity results in an increase in the activity of the Na^+/K^+ ATPase, which when it is present within acidic Golgi compartment of the cells (i.e. trans-Golgi or post-Golgi transport vesicles) disrupts their function. Inhibition of Golgi function results in incomplete processing of virus glycoproteins and/or a failure to efficiently transport these glycoproteins to the cell surface where retrovirus assembly takes place. Inefficient assembly results in over-accumulation of certain viral proteins in infected cells. The presence of drug molecules embedded either in the plasma membrane or in the virus envelope impairs the budding process of the virus, and/or post-release cleavage of the FIV p55 GAG protein into its cleavage products, p24 and p17.

medial Golgi compartments. Treatment with CCX manifested in an accumulation of single and multi-compartmented vacuoles (thought to originate from Golgi apparatus). The origin of these vacuoles could be investigated using anti-p58 antibody in immunogold labelling EM studies. Monensin impairs processing and movements of proteins through the Golgi apparatus, by neutralising the trans-Golgi portion of the organelle (Anderson and Orci, 1988), and a similar mechanism is now proposed to account for the effect of CCX on the Golgi apparatus. A study comparing the effects of CCX and monensin using the DAMP anti-DAMP system will be important to investigate the modes of action of these inhibitors. Drug removal experiments performed in parallel with anti-p58 Golgi antibody labelling immunofluorescent experiments would provide information concerning the rate of function of the Golgi apparatus following drug removal.

The anti-CCX antibody produced in immunised rabbits, needs further testing and characterisation. While the presence of the antibody was detected by an ELISA test, in

4.10. FUTURE WORK.

This study showed that, the triterpenoid compounds impair the function of the Golgi apparatus of CrFK and MDBK cells. It will be important to investigate whether or not this finding also applies to other cell lines. The effect of triterpenoid compounds was investigated using the trans-Golgi compartment specific anti-p58 antibody in immunofluorescence experiments. However, despite repeated attempts this marker failed to label Golgi apparatus in the FeA cell line. Additional Golgi markers, such as wheatgerm agglutinin could also be employed not only to investigate the effect of the drugs on FeA cells, but also to label the cis and medial Golgi compartments. Treatment with CCX manifested in an accumulation of single and multi-compartmented vacuoles (thought to originate from Golgi apparatus). The origin of these vacuoles could be investigated using anti-p58 antibody in immunogold labelling EM studies. Monensin impairs processing and movements of proteins through the Golgi apparatus, by neutralising the trans-Golgi portion of the organelle (Anderson and Orci, 1988), and a similar mechanism is now proposed to account for the effect of CCX on the Golgi apparatus. A study comparing the effects of CCX and monensin, using the DAMP anti-DAMP system will be important to investigate the modes of action of these two drugs. Drug removal experiments performed in parallel with anti-p58 Golgi antibody labelling immunofluorescent experiments would provide information concerning the rate of formation of the Golgi apparatus following drug removal.

The anti-CCX antibody generated in immunised rabbits, needs further testing and characterisation. While the presence of the antibody was detected by an ELISA test, in

immunofluorescence experiments no specific binding was evident. The titre of the putative anti-CCX antibody should be determined.

This study has shown that the triterpenoid compounds have antiviral activity against FeLV-A-, BHV-1, and FIV. However, the experiments with FIV were limited by the low virus titres attained. If the virus could be concentrated it would be possible to perform standard dose-response experiments and so, make a direct comparison between the antiviral activities obtained for FIV and FeLV-A. The unambiguous identification of particular FIV proteins (particularly the env proteins) has been difficult in the work described here; in the future it will be of critical importance to produce either specific or monoclonal antibodies directed against these proteins.

Based on the result of Ito et al., (1987), it has been assumed that the triterpenoid compounds would not interfere with the reverse transcriptase activity of retrovirus- this assumption needs to be confirmed by experiment.

Because, the antiviral activity of the triterpenoid compounds is thought to be mediated through interference with cellular functions , more critically required for virus than cellular growth, it is considered unlikely that drug resistant viruses will emerge. This assumption needs to be confirmed by experiments. Since, ouabain reversed the effect of CCX on the Golgi apparatus, it will be important to determine whether ouabain will modulate the antiviral activity of the triterpenoid compounds.

Triterpenoid compounds and AZT clearly have different modes of action against retroviruses. This fact make it worthwhile to test whether, triterpenoid compounds in combination with RT inhibitors could result in synergistic antiviral activity.

ARAI S. (1981). Activation of human natural killer cells in human defence mechanism (Mizuno, Takeya, Ishida, eds) vol II, pp221-240. University Press, Tokyo. As reported in symposium on prevention of viral hepatitis, 6th International congress of virology (1984), Sendai.

ABE, N., ERINA, T. & ISHIDA, N. (1982). Interferon induction by Glycyrrhizin and Glycyrrhetic acid in mice. *Mic* **102**, 535-539.

REFERENCES

ACKLEY, C.D., YAMAMOTO, J.K., LEVY, N., PEDERSON, N.C. & COOPER, M.D. (1990). Immunoglobulin abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. *Journal of Virology*, **64**, 5652-5655.

ALDOVINI, A. & YOUNG, R.A. (1990). Mutation of RNA and protein sequences involved in human immunodeficiency virus type I packaging result in production of noninfectious virus. *Journal of Virology*, **64**, 1920-1926.

ANDERSON, R.G.W., FALK, J.R., GOLDSTEIN, J.L. & BROWN, M.S. (1984). Visualization of acidic organelles in intact cells by electron microscopy. *Proceedings of the National Academy of Sciences USA*, **81**, 4838-4842.

ANDERSON, R.G.W. & PATHAK, R.K. (1985). Vesicles and cisternae in the trans Golgi apparatus of human fibroblasts are acidic compartments. *Cell*, **40**, 635-643.

ANDERSON, R.G.W. & ORCI, L. (1988). A view of acidic intracellular compartments. *Journal of Cell Biology*, **106**, 539-543.

APPLEYARD, G. (1977). Amantadine-resistance as a genetic marker for influenza viruses. *Journal of General Virology*, **36**, 249-255.

ARYA, S.K., GUO, C., JOSEPHS, S.F. & WONG-STAAL, F. (1985). Transactivator gene of human T-lymphotropic virus type III. *Science*, **229**, 69-73.

BABA, M. & SHIGETA, S.H. (1987). Antiviral activity of glycyrrhizin against varicella-zoster virus *in vitro*. *Antiviral Research*, **7**, 99-107.

BABA, M., MORI, N., SHIGETA, S. & DE CLERCO, E. (1987). Selective inhibitory effect of (S)-9-(3-hydroxy-2-phosphorylmethoxypropyl) adenine and 2'-nor cyclic GMP on adenovirus replication *in vitro*. *Antimicrobial Agents and Chemotherapy*, **31**, 337-339.

BABA, M., PAUWELS, E., BALZAKINI, J., ARNOUT, J., DESMYTER, J. & DE CLERQU, E. (1988). Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus *in vitro*. *Proceedings of the National Academy of Sciences USA*, **85**, 6132-6136.

ARAI, S. (1981). Activation of human natural killer cells in human defence mechanism (Mizmo, Takeya, Ishida, eds) vol II, pp221-240. University Press, Tokyo. As reported in symposium on prevention of viral hepatitis, 6th International congress of virology (1984), Sendai.

ABE, N., EBINA, T. & ISHIDA, N. (1982). Interferon induction by Glycyrrhizin and Glycyrrhetic acid in mice. Microbiology and Immunology, **26**, 535-539.

ACKLEY, C.D., YAMAMOTO, J.K., LEVY, N., PEDERSON, N.C. & COOPER, M.D. (1990). Immunoglobulin abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. Journal of Virology, **64**, 5652-5655.

ALDOVINI, A. & YOUNG, R.A. (1990). Mutation of RNA and protein sequences involved in human immunodeficiency virus type I packaging result in production of noninfectious virus. Journal of Virology, **64**, 1920-1926.

ANDERSON, R.G.W., FALK, J.R., GOLDSTEIN, J.L. & BROWN, M.S. (1984). Visualization of acidic organelles in intact cells by electron microscopy. Proceedings of the National Academy of Sciences USA, **81**, 4838-4842.

ANDERSON, R.G. W. & PATHAK, R.K. (1985). Vesicles and cisternae in the trans Golgi apparatus of human fibroblasts are acidic compartments. Cell, **40**, 635-643.

ANDERSON, R.G.W. & ORCI, L. (1988). A view of acidic intracellular compartments. Journal of Cell Biology, **106**, 539-543.

APPLEYARD, G. (1977). Amantadine-resistance as a genetic marker for influenza viruses. Journal of General Virology, **36**, 249-255.

ARYA, S.K., GUO, C., JOSEPHS, S.F. & WONG-STAAAL, F. (1985). Transactivator gene of human t-lymphotropic virus type III. Science, **229**, 69-73.

BABA, M. & SHIGETA, S.H. (1987). Antiviral activity of glycyrrhizin against varicella-zoster virus *in vitro*. Antiviral Research, **7**, 99-107.

BABA, M., MORI, S., SIGETA, S. & DE CLERCQ, E. (1987). Selective inhibitory effect of (S)-9-(3-hydroxy-2-phosphorylmethoxypropyl) adenine and 2'-nor cyclic GMP on adenovirus replication *in vitro*. Antimicrobial Agents and Chemotherapy, **31**, 337-339.

BABA, M., PAUWELS, R., BALZARINI, J., ARNOUT, J., DESMYTER, J. & DE CLERCQ, E. (1988). Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus *in vitro*. Proceedings of the National Academy of Sciences USA, **85**, 6132-6136.

BISCHOFBERGER, N. WAGNER, R.W. (1992). Antisense approaches to antiviral agents. Seminars in Virology, **3**, 57-66.

BABIUK, L.A., L'ITALIEN, J., VAN DRUNEN LETTEL-VAN DEN HURK, S., ZAMB, T., LAWMAN, M.J.P., HUGHES, G. & GIFFORD, G.A. (1987). Protection of cattle from bovine herpesvirus type 1 (BHV-1) infection by immunization with individual viral glycoproteins. Virology, **159**, 57-66.

BACHALERIE, F., ALCAMI, J., HAZAN, V., ISRAEL, N., GOUD, B., ARENZANA, SIESDEDOS, F. & VIRELIZIER, J.L. (1990). Constitutive expression of the human immunodeficiency virus Nef protein does not influence basal or induced HIV-LTR activity. Journal of Virology, **64**, 3059-3062.

BACON, T.H. & BOYD, M.R. (1995). Activity of penciclovir against Epstein-Barr virus. Antimicrobial Agents and Chemotherapy, **39**, 1599-1602.

BALFOUR, H.H. (1988). Acyclovir. Antimicrobial Agents Annual, **3**, 345-360.

BALZARINI, J., HAO, Z., HERDEWIJN, P., JOHNS, D.G. & DE CLERCQ, E. (1991). Intracellular metabolism and mechanism of anti-retrovirus action of 9-(2-phosphonylmethoxyethyl)adenine, a potent anti-human immunodeficiency virus compound. Proceedings of the National Academy of Sciences USA, **88**, 1499-1503.

BARON, D.N. & GREEN, R.J. (1986). Action of compounds with effective *in vivo* mineralocorticoid activity on ion transport in leucocytes. British Journal of Clinical Pharmacology, **21**, 27-34.

BEEBE, A.M., DUA, N., FAITH, T.G., MOORE, P.F., PEDERSON, N.C. & DANDEKAR, S. (1994). Primary stage of feline immunodeficiency virus infection: viral dissemination and cellular targets. Journal of Virology, **68**, 3080-3091.

BENVENISTE, R.E., SHERR, C.J. & TODARO, G.J. (1975). Evolution of type C viral genes: origin of feline leukemia virus. Science, **190**, 886-888.

BENDINELLI, M., PISTELLO, M., LOMBARDI, S., POLL, A., GARZELLI, C., MATEUCCI, D., CECCHERINI-NELLI, L., MALVALDI, G. & TOZZINI. (1995). Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. Clinical Microbiology Review, **8**, 87-112.

BERTORELLO, A.M., APERIA, A., WALAAS, S.I., NAIRN, A.C. & GREENGARD, P. (1991). Phosphorylation of the catalytic subunit of Na⁺, K⁺-ATPase inhibits the activity of the enzyme. Proceedings of the National Academy of Sciences USA, **88**, 11359-11362.

BEUTNER, K.R. (1995). Valciclovir: a review of its antiviral activity, pharmacokinetic properties, and clinical efficacy. Antiviral Research, **28**, 281-290.

BISCHOFBERGER, N. WAGNER, R.W. (1992). Antisense approaches to antiviral agents. Seminars in Virology, **3**, 57-66.

BLUM, M.R., SOUL-LAWTON, J., SMITH, C.M., ON, N.T., POSNER, J. & ROLAN, P.E. (1994). Increased bioavailability of acyclovir from oral valciclovir in healthy volunteers. Antiviral Research, **23**, 74 (Abstr).

BOYD, M.R., BACON, T.H., SUTTON, D. & COLE, M. (1987). Antiherpesvirus activity of 9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine (BRL 39123) in cell culture. Antimicrobial Agents and Chemotherapy, **31**, 1238-1242.

BOYD, M.R., BACON, T.H. & SUTTON, D. (1988). Antiherpesvirus activity of 9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine (BRL 39123) in animals. Antimicrobial Agents and Chemotherapy, **32**, 358-363.

BOWEN, E.F., ATKINS, M., WELLER, I.V.D. & JOHNSON, M.A. Current clinical treatment of retroviral infections. In antiviral Chemotherapy, edited by **D.J., JEFFRIES, AND E. DE CLERCQ**, 1995, PAGE 73.

BIRON, K.K., & ELION, G. (1980). *In vitro* susceptibility of varicella-zoster virus to acyclovir. Antimicrobial Agents and Chemotherapy, **18**, 443-447.

BRONSON, J.J., HO, H-T., De BOECK, H., WOODS, K., GHAZZOULI, I., MARTIN, J.C. & HITCHCOCK, M.J.M. (1990). Biochemical pharmacology of acyclic nucleotide analogs. Annual New York Academy of Sciences, **616**, 398-407.

BRONSON, J.J., FERRARA, L.M., HITCHCOCK, M.J.M., HO, H., WOODS, K.L., GHAZZOULI, I., KERN, E.R., SOIKE, K.F. & MARTIN, J.C. (1991). (S)-1-(3-hydroxy-2-(phosphonylmethoxy)peopyl)cytosine (HPMPC): a potent antiherpesvirus agent, p.277-283. In C.Lopez et al., (ed.), Immunobiology and prophylaxis of human herpesvirus infections. Plenum Press, New York.

BROWN, E.W., YUHKEI, N., PACKER, C. & OBRINE, S.J. (1994). A lion lentivirus related to feline immunodeficiency virus: epidemiologic and phylogenetic aspects. Journal of Virology, **68**, 5953-5968.

BROWN, M.S., ANDERSON, R.G.W. & GOLDSTEIN, J.L. (1983). Recycling receptors: The round trip itinerary of migrant membrane proteins. Cell, **32**, 663-667.

BROWN, W.C., BISSEY, L., LOGAN, K.S., PEDERSON, N.C., ELDER, J.H. & COLLISON, E.W. (1991). Feline immunodeficiency virus infects both CD4⁺ and CD8⁺ T lymphocytes. Journal of Virology, **65**, 3359-3364.

BRUNNER, D. & PEDERSON, N.C. (1989). Infection of peritoneal macrophages *in vitro* and *in vivo* with feline immunodeficiency virus. Journal of Virology, **63**, 5483-5488.

CARRINGTON, D. (1994). Prospects for improved efficacy with antiviral prodrugs: will valacyclovir and famciclovir meet the clinical challenge. The International Antiviral News, 2, 50-53

BUKRINSKAYA, A.G., VORKUNOVA, N.E. & PUSHKARSKAYA, N.L. (1982). Uncoating of a rimantadine-resistant variant of influenza virus in the presence of rimantadine. Journal of General Virology, **60**, 61-66.

BUKRINSKAYA, A.G., VORKUNOVA, N.K., KORNILAYEVA, G.V., NAMANBETOVA, R.A. & VORKUNOVA, G.K. (1982a). influenza virus uncoating in infected cells and effects of rimantadine. Journal of General Virology, **60**, 49-59.

BURNS W.H., SARAL, R., SANTOS, G.W., LASKIN, O.L., LEITMAN, P.S., McLAREN, C. & BARRY, D.W. (1982). Isolation and characterisation of resistant herpes simplex virus after acyclovir therapy. Lancet, **1**, 421.

CAIN, C.C., SIPE, D.M. & MURPHY, R.F. (1989). Regulation of endocytic pH by the Na^+ , K^+ -ATPase in living cells. Proceedings of the National Academy of Sciences USA, **86**, 544-548.

CARPENTER, D.E. & MISRA, V. (1991). The most abundant protein in bovine herpes 1 virions is a homologue of herpes simplex virus type 1 UL47. Journal of General Virology, **72**, 3077-3084.

CHAUDHURY, V.K., MIZUKAMI, T. & FUERST, T.R. (1988). Selective killing of HIV infected cells by recombinant human CD4-pseudomonas exotoxin in hybrid protein. Nature, **335**, 369-372

CHENG-MAYER, C., WEISS, C., SETO, D. & LEVY, J.A. (1989). Isolates of human immunodeficiency virus type-1 from the brain may constitute a special group of the AIDS virus. Proceedings of the National Academy of Sciences USA, **86**, 8575-8579.

CHEESEMAN, S.H., HATTOX, S.E., McLAUGHLIN, M.M., KOUP, R.A., ANDREWS, C., BOVA, C.A., PAR, J.W., ROY, T., SULLIVAN, J.L. & KEIRNS, J.J. (1993). Pharmacokinetics of nevirapine: Initial single-rising-dose study in humans. Antimicrobial Agents and Chemotherapy, **37**, 178-182.

CHRISP, P.L. & CLISSOLD, S.P. (1991). Foscarnet. Drugs, **41**, 104-129.

CLEMENTS, G.J., PRICE-JONES, M.J., STEPHENS, P.E., SUTTON, C., SCHULTZ, T.F., CLAPHAM, P.R., McKEATING, J.A., McLURE, M.O., THOMSON, S., MARSH, M., KAY, J., WEISS, R.A. & MOORE, J.P. (1991). The V3 loops of HIV-1 and HIV-2 surface glycoproteins contain proteolytic cleavage sites. A possible function in viral fusion. AIDS Research and Human Retroviruses, **7**, 3-16.

COEN, D.M. & SCHAFFER, P.A. (1980). Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1. Proceedings of the National Academy of Sciences USA, **77**, 2265-2269.

COEN, D.M., FURMAN, P.A., GELEP, P.T. & SCHAFFER, P.A. (1982). Mutations in the herpes simplex virus DNA polymerase gene can confer resistance to 9- β -d arabinofuranosyladenine. Journal of Virology, **41**, 909-918.

COHEN, E.A., GAUDREAU, P., BRAZEAU, P. & LANGELIER, Y. (1986). Specific inhibition of herpesvirus ribonucleotide reductase by a nonapeptide derived from the carboxy terminus of subunit 2. Nature, **321**, 441-443.

COLICELLI, J. & GOFF, S.P. (1988). Sequence and spacing requirements of a retrovirus integration site. Journal of Molecular Biology, **199**, 47-59.

COLLINS, J.K., BUTCHER, A.C., RIEGEL, C.A., McGRANE, V., BLAIR, C.D., TERAMOTO, Y.A. & WINSTON, S. (1984). Neutralizing determinants defined by monoclonal antibodies on polypeptides specified by bovine herpesvirus 1. Journal of Virology, **52**, 403-409.

COLLINS, P. (1983). The spectrum of antiviral activities of acyclovir *in vitro* and *in vivo*. Journal of Antimicrobial Chemotherapy, **12**, 19-27.

CONCORDE COORDINATING COMMITTEE. (1994). Concord: M RC/ANRS randomised double-blind controlled trial of immediate and deferred zidovudine in symptome-free HIV infection. Lancet, **343**, 871-881.

CONDRA, J.H., SCHIEF, W.A., BLAHY, O.M., GABRYELSKI, L.J., GRAHAM, D.J., QUINTERO, J.C., RHODES, A., ROBBINS, H.L., ROTH, E., SHIVAPRAKASH, M., TITUS, D., YANG, T., TEPLER, H., SQUIRES, E., DEUTCH, P.J. & EMINI, E.A. (1995). *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature, **374**, 569-571.

CONN, J.C., ROVNER, D.R. & COHEN, E.L. (1968). Licorice induced psuedoaldosteronism. Journal of American Medical Association, **205**, 492-496.

COONEY, D.A., DALAL, M., MITSUYA, H.M., McMAHON, J.B., NADKARNI, H., BALZARINI, J., BRODER, S. & JOHNS, D.G. (1986). Initial studies on the cellular pharmacology of 2', 3', -dideoxycytidine, an inhibitor of HTLV-III infectivity. Biochemistry and Pharmacology, **35**, 2065-2068.

CRANCE, J.M., LEVQUE, F., CHOUSTERMAN, S., JOUAN, A., TREPO, C. & DELOINCE, R. (1995). Antiviral activity of recombinant interferon-alpha on hepatitis A virus replication in human liver cells. Antiviral Research, **28**, 69-80.

CRONN, R.C., WHITMER, J.D. & NORTH, T.W. (1992). RNase H activity associated with reverse transcriptase from feline immunodeficiency virus. Journal of Virology, **66**, 1215-1218.

CRUMPACKER, C.S. (1992). Antiviral therapy for human immunodeficiency virus type 1. Seminars in Virology, **3**, 13-20.

CSONKA, G.W. & TYRRELL, D.A. (1984). Treatment of herpes genitalis with carbenoxolone and cicloxolone creams: A double blind placebo controlled clinical trial. British Journal of Venereal Disease, **60**, 178-181.

CULLEN, B.R., HAUBER, J., CAMBELL, K., SODROSKI, J.G., HASELTINO, W.A., & ROSEN, C.A. (1988). Subcellular localisation of the transacting *art* gene product. Journal of Virology, **62**, 2498-2501.

CULLEN, B.R. (1990). The HIV-1 TAT protein an RNA sequence specific processivity factor? Cell, **63**, 655-657.

CULLEN, B.R. (1991). Regulation of human immunodeficiency virus replication. Annual Review of Microbiology, **45**, 219-250.

CUNDY, K.C., BARDITCH-CROVO, P., WALKER, R.E., COLLIER, A.C., EBELING, D., TOLLE, J. & JAFFE, H.S. (1995). Clinical pharmacokinetics of adefovir in human immunodeficiency virus type 1-infected patients. Antimicrobial agents and Chemotherapy, **39**, 2401-2405.

DANIELS, R.S., DOWNIE, J.C., HAY, A.J., KNOSSOW, M., SKEHEL, J.J., WANG, M.L. & WILEY, D.C. (1985). Fusion mutants of the influenza virus hemagglutinin glycoprotein. Cell, **40**, 431-439.

DARGAN, D.J. & SUBAK-SHARPE, J.H. (1985). The effect of triterpenoid compounds on uninfected herpes simplex virus-infected cells in culture. I. Effect on cell growth, virus particles and virus replication. Journal of General Virology, **66**, 1771-1784.

DARGAN, D.J. & SUBAK-SHARPE, J.H. (1986). The effect of triterpenoids compounds on uninfected and herpes simplex virus-infected cells in culture. II. DNA and protein synthesis, polypeptide processing and transport. Journal of General Virology, **67**, 1831-1850.

DARGAN, D.J. & SUBAK-SHARPE, J.H. (1986a). The antiviral activity against herpes simplex virus of the triterpenoid compound carbenoxolone sodium and cicloxolone sodium. Journal of Antimicrobial Chemotherapy, **18**, supplement B, 185-200.

DARGAN, D.J., AITKEN, J.D. & SUBAK-SHARPE, J.H. (1988). The effect of triterpenoid compounds on uninfected and Herpes Simplex virus-infected cells in culture. III. Ultrastructural study of virion maturation. Journal of General Virology, **69**, 439-444.

DARGAN, D.J. & SUBAK-SHARPE. (1991). The difference in sensitivity to cicloxolone sodium between herpes simplex virus types 1 and 2 maps to the location of genes UL22 (gH) and UL44 (gC). Journal of General Virology, **72**, 377-384.

DOLIN, R., REICHMAN, R.C., MADORE, H.P., MAYNARD, R., LINTON, P.N. & WEBBER-JONES, J. (1982). A controlled trial of amantadine and rimantadine in the treatment of influenza A virus infections. Journal of General Virology, **73**, 407-411.

DARGAN, D.J., GALT, C.B. & SUBAK-SHARPE, J.H. (1992a). The effect of cicloxolone sodium on the replication of vesicular stomatitis virus in BSC-1 cells. Journal of General Virology, **73**, 397-406.

DARGAN, D.J. & SUBAK-SHARPE, J.H. (1992b). The antiviral activity of triterpenoid compounds. Seminars in Virology, **3**, 31-40.

DAVIES, W.L., GRUNERT, R.R., HAFF, R.F., McGAHEN, J.W., NEUMAYER, E.M., PAULSHOCK, M., WATTS, J.C., WOOD, T.R., HERMAN, E.C. & HOFFMAN, C.E. (1964). Antiviral activity of 1-adamantanamine (amantadine). Science, **144**, 862-863.

DEBYSER, Z., PAUWELS, R., ANDRIES, K., DESMYTER, J., KUKALA, M., JANSSEN, P.A.J. & DE CLERCQ, E. (1991). An antiviral target on reverse transcriptase of human immunodeficiency virus type 1 revealed by tetrahydroimidazo-[4, 5, 1-jk] [1,4]benzodiazepine (1H)-one and thione derivatives. Proceedings of the National Academy of Sciences USA, **88**, 1451-1455.

De CLERCQ, E., HOLY, A., ROSENBERG, I., SAKUMA, T., BALZARINI, J. & MAUDGAL, P.C. (1986). A novel selective broad spectrum anti-DNA virus agent. Nature, **323**, 464-466.

DE CLERCQ, E. (1991). Broad-spectrum anti-DNA virus and anti-retrovirus activity of phosphonylmethoxyalkylpuirnes and pyrimidines. Biochemistry and Pharmacology, **42**, 963-972.

De MIRANDA, P. & BLUM, M.R. (1983). Pharmacokinetics of acyclovir after intravenous and oral administration. Journal of Antimicrobial Chemistry, **12**, 29-37.

DIGARD, P., WILLIAMS, K.P., HENSLEY, P., BROOKS, I.S., DAHL, C. & COEN, D.M. (1995). Specific inhibition of herpes simplex virus DNA polymerase by helical peptides corresponding to the subunit interface. Proceedings of the national Academy of Sciences USA, **92**, 1456-1460.

DIVITA, G., RESTLE, T., GOODY, R.S., CHERMANN, J.C. & BAILLON, J.G. (1994). Inhibition of human immunodeficiency virus type 1 reverse transcriptase dimerization using synthetic peptides derived from connection domain. The Journal of Biological Chemistry, **269**, 13080-13083.

DOLIN, R., REICHMAN, R.C., MADORE, H.P., MAYNARD, R., LINTON, P.N. & WEBBER-JONES, J. (1982). A controlled trial of amantadine and rimantadine in the prophylaxis of influenza A infection. New England Journal of Medicine, **307**, 580-584.

DONAHUE, P.R., HOOVER, E.A., BELTZ, G.A., RIEDEL, N., HIRSCH, V.M., OVERBAUGH, J. & MULLINS, J.I. (1988). Strong sequence conservation among horizontally transmissible, minimally pathogenic feline leukemia viruses. Journal of Virology, **62**, 722-731.

DONAHUE, P.R., QUACKENBUSH, S.L., GALLO, M.V., DENORONHA, C.M.C., OVERBAUGH, J., HOOVER, E.A. & MULLINS, J.I. (1991). Viral genetic determinants of T-cell killing and immunodeficiency disease induction by the feline leukemia virus FeLV-FAIDS. Journal of Virology, **65**, 4461-4469.

DORNSIFE, R.E., ST. CLAIR, M.H., HUANG, A.T., PANELLA, T.J., KOSZALKA, G.W., BURNS, C.L. AVERETT, D.R. (1991). Anti-human immunodeficiency virus synergism by zidovudine (3'-azidothymidin) and didanosine(dideoxyinosine) contrasts with their additive inhibition of normal human progenitor cells. Antimicrobial Agents and Chemotherapy, **35**, 322-328.

DOUGLAS, R.G. (1990). Prophylaxis and treatment of influenza. New England Journal of Medicine, **322**, 443-450.

DOW, S.W., DRIETZ, M.J. & HOOVER, E.A. (1992). Feline immunodeficiency virus neurotropism: evidence that astrocytes and microglia are the primary target cells. Veterinary Immunology and Immunopathology, **35**, 23-35.

EARNSHAW, D.L., BACON, T.H., DARLISON, S.J., EDMONDS, K., PERKINS, R.M. & VERE HODGE, R.A. (1992). Mode of antiviral action of penciclovir in MRC-5 cells infected with herpes simplex virus type 1 (HSV-1), HSV-2, and varicella zoster virus. Antimicrobial Agents chemotherapy, **36**, 2747-2757.

EBERLE, J., BECHOWSKY, B., ROSE, D., HAUSER, U., VON DER HELM, K., GURTNER, L. & NITSCHKO, H. (1995). Resistance of HIV type 1 proteinase to Ro 31-8959. AIDS Research and Human Retroviruses, **11**, 671-676.

EGBERINK, H., BORST, M., NIPHUIS, H., BALZARINI, J., NEU, H., SCHELLEKENS, H., DE CLERCQ, E., HORZINEK, M. & KOOLEN, M. (1990). Suppression of feline immunodeficiency virus infection *in vivo* by 9-(2-phosphonomethoxyethyl)adenine. Proceedings of the National Academy of Sciences USA, **87**, 3087-3091.

EGBERINK, H.F., EDERVEEN, J., MONTERLARO, R.C., PEDERSON, N. C., HORZINEK, M. C. & KOOLEN, M.J.M. (1990a). Intracellular proteins of feline immunodeficiency virus and their antigenic relationship with equine infectious anaemia virus proteins. Journal of General Virology, **71**, 739-743.

FELBER, B.K., DRYSDALE, C.M. & PAVLATIS, G.N. (1990). Feedback.
ELBEIN, A.D., MITCHELL, M., SANFORD, B.A., FELLOWS, L.E. & EVANS, S.V. (1984). The pyrrolidine alkaloid 2,5-dihydroxymethyl-3, 4, dihydroxypyrrolidine inhibits glycoprotein processing. Journal of Biological Chemistry, **259**, 12409-12413.

FIELD, A.K. & BIRON, K.E. (1994). "The end of resistance" revisited: resistance of
ELDER, J.H., LERNER, D.L., HASSELKUS-LIGHT, C.S., FONTENOTO, D.J., HUNTER, E., LUCIW, P.A., MONTELARO, R.C. & PHILLIPS, T.R. (1992). Distinct subsets of retroviruses encode dUTPase. Journal of Virology, **66**, 1791-1794.

ELDER, J.H., SCHNOLZER, M., HASSELKUS-LIGHT, C.S., HENSON, M., LERNER, D.A., PHILLIPS, T.R., WAGAMAN, P.C. & KENT, S.B.H. (1993). Identification of proteolytic processing sites within the GAG and POL polyproteins of feline immunodeficiency virus. Journal of Virology, **67**, 1869-1876.

EMERMAN, M., VAZEUX, R. & PEDER, K. (1989). The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localisation. Cell, **57**, 1155-1165.

EPSTEIN, M.T., ESPINER, E.A., DONALD, R.A. & HUGHES, H. (1977). Effect of eating liquorice on the renin-angiotensin aldosterone axis in normal subjects. British Medical Journal, **1**, 488-490.

ESSEX, M. (1975). Horizontally and vertically transmitted oncoviruses of cats. Advance Cancer Research, **21**, 175-248.

ESTES, J. & HUANG, E.-S. (1977). Stimulation of cellular thymidine kinase by human cytomegalovirus. Journal of Virology, **24**, 13-21.

FAN-HAVARD, P., NAHATA, M.C. & BRADY, M.T. (1989). Ganciclovir: a review pharmacology, therapeutics efficacy and potential use for treatment of congenital cytomegalovirus infection. Journal of Clinical Pharmacology and Therapeutic, **14**, 329-340.

FEHLER, F., HERRMANN, J.M., SAALMULLER, A., METTENLEITER, T.C. & KEIL, G. (1992). Glycoprotein IV of bovine herpesvirus 1-expression cell line complements and rescues a conditionally lethal viral mutant. Journal of Virology, **66**, 831-839.

FEINBERG, M.B., JARRETT, R.F., ALDOVINI, A., GALLO, R.C. & WONG-STAAAL, F. (1986). HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell, **46**, 807-817.

FEINBERG, M.B., BAITIMORE, D. & FRANKEL, A.D. (1991). The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. Proceedings of the National Academy of Sciences USA, **88**, 4045-4049.

- FELBER, B.K., DRYSDALE, C.M. & PAVLAKIS, G.N.** (1990). Feedback regulation of human immunodeficiency virus type I expression by the REV protein. Journal of Virology, **64**, 3734-3741.
- FIELD, A.K. & BIRON, K.E.** (1994). "The end of innocence" revisited: resistance of herpesvirus to antiviral drugs. Clinical Microbiology Review, **7**, 1-13.
- FIELD, H.J., McMILLAN, A. & DARBY, G.** (1981). The sensitivity of acyclovir-resistant mutants of herpes simplex virus to other antiviral drugs. Journal of Infectious Disease, **143**, 281-285.
- FIELD, H.J., TEWARI, D., SUTTON, D. & THACKRAY, A.M.** (1995). Comparison of efficacies of famciclovir and valciclovir against herpes simplex virus type 1 in a murine immunosuppression model. Antimicrobial Agents and Chemotherapy, **39**, 1114-1119.
- FISCHL, M.A., RICHMAN, D.D., GRIECO, M.H., GOTTLIEH, M.S., VOLBERDING, P.A., LASKIN, O.A., LEEDOM, J.M., GROOPMAN, J.E., MILDVAN, D., SCHOOLEY, R.T., JACKSON, G.G., DURACK, D.T., KING, D. & THE AZT COLLABORATIVE WORKING GROUP.** (1987). The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex: a double-blind, placebo-controlled trial. New England Journal of Medicine, **317**, 185-191.
- FISHER, A.G., ENSOLI, B., IVANOF, L., CHAMBERLIN, M., PETTEWAY, S., RATNER, L., GALLO, R.C. & WONG-STAAAL, F.** (1987). The sor gene of HIV-1 is required for efficient virus transmission in vitro. Science, **237**, 888-893.
- FISHKES, H. & RUDNICK, G.** (1982). Bioenergetics of serotonin transport by membrane vesicles derived from platelet dense granules. Journal of Biological Chemistry, **257**, 5671-5677.
- FLETCHER, C.V., COLLIER, A.C., RHAME, F.S., BENNET, D., PARA, M.F., BEATY, C.C., JONES, C.E. & BALFOUR, H.H.** (1994). Foscarnet for the suppression of human immunodeficiency virus replication. Antiviral Agents and Chemotherapy, **38**, 604-607.
- FRANK, K.B., NOLL, G.J., CONNELL, E.V. & SIM, I.S.** (1991). Kinetic interaction of human immunodeficiency virus type 1 reverse transcriptase with antiviral tetrahydroimidazo[4, 5, 1-jk]-[1, 4]-benzodiazepine-2-(1H)-thione compound, R82150. The Journal of Biological Chemistry, **266**, 14232-14236.
- FRANKEL, A.D. & PABO, C.O.** (1988). Cellular uptake of the tat protein from human immunodeficiency virus. Cell, **55**, 1189-1194.

FREED, E.O., MYERS, D.J. & RISSER, R. (1990). Characterisation of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proceedings of the National Academy of Sciences USA, **87**, 4650-4654.

FUCHS, R., SCMID, S. & MELLMAN, I. (1989). A possible role for Na⁺, K⁺-ATPase in regulating ATP-dependent endosome acidification. Proceedings of the National Academy of Sciences USA, **86**, 539-543.

FUJISAWA, K., WATANABE, Y. & KIMURA, K. (1980). Therapeutic approach to chronic active hepatitis with glycyrrhizin. Asian Medical Journal, **23**, 745-756.

FUJISAWA, K. (1993). What is Stronger Neo-minophagen C pp1-2. As reported in the satellite symposium on therapeutic approach to the chronic active liver disease. The 1993 international symposium on viral hepatitis and liver disease Tokyo.

FULTON, R., PLUMB, M., SHIELD, L. & NEIL, J. (1990). Structural diversity and nuclear protein binding sites in the long terminal repeats of feline leukemia virus. Journal of Virology, **64**, 1675-1682.

FURMAN, P.A., ST. CLAIR, M.H. & SPECTOR, T. (1984). Acyclovir triphosphate is a suicide inactivator of the herpes simplex virus DNA polymerase. Journal of Biological Chemistry, **259**, 9575-9579.

FURMAN, P.A., FYFE, J.A., ST. CLAIR, M.H., WEINHOLD, K., RIDEOUT, J.L., FREEMAN, G.A., NUSINOFFLEHRMAN, S., BOLOGNESI, D.P., BRODER, S., MITSUYA, H. & BARRY, D.W. (1986). Phosphorylation of 3'-azido-3'-azido-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. Proceedings of the National Academy of Sciences, **83**, 8333-8337.

FURMANN, U., BAUSE, E. & PLOEGH, H. (1985). Inhibitors of oligosaccharide processing. Biochemical and Biophysical ACTA, **825**, 95-100.

FYFE, J.A., KELLER, P.M., FURMAN, P.A., MILLER, R.L. & ELBION, G.B. (1978). Thymidine kinase from herpes simplex virus Phosphorylates the new antiviral compound 9- (2-hydroxyethoxymethyl) guanine. Journal of Biological Chemistry, **253**, 8721-8727.

GALT, C., DARGAN, D.J. & SUBAK-SHARPE, J.H. (1990). *In vitro* studies of the antiviral range of cicloxolone sodium and identification of cell lines tolerant to the drug. Antiviral Chemistry and Chemotherapy, **1** (2), 115-123.

GARCIA, J.V. & MILLER, A.D. (1991). Serine phosphorylation independent down regulation of cell surface CD4 by Nef. Nature, **350**, 508-511.

GARRY, R.F., WESTBROOK, K. & WAITE, M.R.F. (1979). Differential effects of ouabain on host- and Sindbis virus-specified protein synthesis. Virology, **99**, 179-182.

GILBERT, B.E. & KNIGHT, V. (1986). Biochemistry and clinical applications of ribavirin. Antimicrobial Agents and Chemotherapy, **30**, 201-205.

GLICKMAN, J., CROEN, K., KELLY, S. & AL-AWAQATI, Q. (1983). Golgi membranes contain an electrogenic H⁺ pump in parallel to a chloride conductance. Journal of Cell Biology, **97**, 1303-1308.

GORELICK, R.J., HENDERSON, L.E., HASER, J.P. & REIN, A. (1988). Point mutants of molony murine leukemia virus that fail to package viral RNA: evidence for specific RNA recognition by a "zink finger like" protein sequence. Proceedings of the National Academy of Sciences, USA **85**, 8420-8424.

COTTER, S.M. (1992). Feline leukemia virus pathophysiology, prevention, and treatment. Cancer Investigation, **10**, 173-181.

GOTTLINGER, H.G., SODROSKI, J.G. & HASELTINE, W.A. (1989). Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type I. Proceedings of the National Academy of Sciences USA, **86**, 5781-5785.

GREENE, W.C. & CULLEN, B.R. (1990). The rev-rex connection: convergent strategies for the post transcriptional regulation of HIV-1 and HTLV-1 gene expression. Seminars in Virology, **1**, 195-204.

GREEN, W.K., MEERS, J. DEL FIERRO, G. CARNEGIE, P.R. & ROBINSON, W.F. (1993). Extensive sequence variation of feline immunodeficiency virus env genes in isolates from naturally infected cats. Archives of Virology, **133**, 51-62.

GRUTERS, R.A., NEEFJES, J.J., TERSMETTE, M., DE GOEDE, TULP, A., HUISMAN, H.G., MIEDEMA, F. & PLOEGH, H.L. (1987). Interference with HIV-induced syncytium formation and viral infectivity by inhibition of trimming glucosidase. Nature, **330**, 74-77.

GUY, B., KIENY, M.P., RIVIERE, Y., Le PEUCH, C., DOTT, K., GIRARD, M., MONTAGNIER, L. & Le COCQ, J.P. (1987). HIV F/3' ORF encodes a phosphorylated GTP binding protein resembling an oncogene product. Nature, **330**, 266-269.

GUYADER, M., EMERMAN, M., MONTAGNIER, L. & PEDEN, K. (1989). VPX mutants of HIV-2 are infectious in established cell lines but display a severe defect in peripheral blood lymphocytes. EMBO Journal, **8**, 1169-1175.

HAN, L., YUN, J.S. & WAGNER, T.E. (1991). Inhibition of molony murine leukemia virus-induced leukemia in transgenic mice expressing antisense RNA complementary to the retroviral packaging sequences. Proceedings of the National Academy of Sciences USA, **88**, 4313-4317.

HARDY, W.D. Jr., OLD, A.J., HESS, P.W., ESSEX, M. & COTTER, S.M. (1973). Horizontal transmission of feline leukemia virus. Nature, **244**, 266-269.

HARDY, W.D. Jr., HESS, P.W., MACEWEN, E.G., MCCLELLAND, A.J., ZUKERMAN, E.E., ESSEX, M., COTTER, S.M. & JARRETT, O. (1976). Biology of feline leukemia virus in the natural environment. Cancer Research, **36**, 582-588.

HARRIS, M., HISLOP, S., PATSILNACOS, P. & NEIL, J.C. (1992). *In vivo* derived HIV-1 nef gene products are heterogeneous and lack detectable nucleotide binding activity. AIDS Research and Human Retroviruses, **8**, 537-543.

HATTORI, T., IKEMATSU, S., KOITO, A., MATSUSHITA, S., MAEDA, Y., HADA, M., FUJIMAKI, M. & TAKATSUKI, K. (1989). Preliminary evidence for inhibitory effect of glycyrrhizin on HIV replication in patients with AIDS. Antiviral Research, **11**, 255-262.

HAY, A.J., WOLSTENHOLME, A.J., SKEHEL, J.J. & SMITH, M.H. (1985). The molecular basis of the specific anti-influenza action of amantadine. EMBO Journal, **4**, 3021-3024.

HAY, A.J. (1992). The action of adamantanes against influenza A viruses: inhibition of the M2 ion channel protein. Seminars in Virology, **3**, 21-30.

HELENIUS, A., MARSH, M. & WHITE, J. (1982). Inhibition of semliki forest virus penetration by lysomotropic weak bases. Journal of General Virology, **58**, 47-61.

HENDERSON, L.E., SOWDER, R.C., CPELAND, T.D., BENVENISTE, R.E. & ORSZLAN, S. (1988). Isolation and characterisation of a novel protein (X-ORF product) from SIV and HIV-2. Science, **241**, 199-201.

HO, H.-T., WOODS, K.L., BRONSON, J.J., DE BOECK, (1988). Intracellular metabolism of the antiherpes agent (S)-1[3-hydroxy-2(phosphonyl-methoxy)propyl]cytosine on lymphocyte transformation and delayed-type hypersensitivity responses. Molecular Pharmacology, **41**, 197-202.

HO, D.D., NEUMANN, A.V., PEREISON, A.S., CHEN, W., LEONARD, J.M. & MARKOWITZ, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London), **373**, 123-126.

HOFFMANN-FEZER, G., THUM, J., ACKLEY, C., HERBOLD, M., MYSLIWETZ, J., THEFELD, S., HARTMANN, K., & KRAFT, W. (1992). Decline in CD4⁺ cell numbers in cats with naturally acquired feline immunodeficiency virus infection. Journal of Virology, **66**, 1484-1488.

HORWITZ, J.P., CHUA, J. & NOEL, M. (1964). Nucleoside V. The monomesylates of 1-(2,-deoxy-B-d-lyxofuranosyl) thymine. Journal of Organic Chemistry, **29**, 2076-2078.

HOSIE, M. J., ROBERTSON, C. & JARRETT, O. (1989). Prevalence of feline leukemia virus and feline immunodeficiency virus in cats in the United Kingdom. Veterinary Research, **128**, 293-297.

HOSIE, M.J. & JARRETT, O. (1990). Serological responses of cats to feline immunodeficiency virus. AIDS, **4**, 215-220.

HOSIE, M., WILETT, B.J., DUNSFORD, T.H., JARRETT, O., & NIEL, J. (1993). A monoclonal antibody which blocks infection with feline immunodeficiency virus identifies a possible non-CD4 receptor. Journal of Virology, **67**, 1667-1671.

HSU, M-C., SCHUTT, A.D., HOLLY, M., SLICE, L.W., SHERMAN, M.I., RICHMAN, D.D., POTASH, M.J. & VOLSKY, D.J. (1991). Inhibition of HIV replication in acute and chronic infection *in vitro* by a Tat antagonist. Science, **254**, 1799-1802.

HSU, M-C., DHINGRA, U., EARLY, J.V., HOLLY, M., KIETH, D., NALIN, C.M., RICHOU, A.R., SHUTT, A.D., TAM, S.Y., POTASH, M.J., VOLSKY, D.J. & RICHMAN, D.D. (1993). Inhibition of type 1 human immunodeficiency virus replication by a Tat antagonist to which the virus remains sensitive after prolonged exposure *in vitro*. Proceedings of the National Academy of Sciences USA, **90**, 6395-6399.

HU, W.S. & TEMIN, H.M. (1990). Retroviral recombination and transcription. Science, **250**, 1227-1233.

ITO, M., NAKASHIMA, H., BABA, M., PAUWELS, R., DE CLERCQ, E., SHIGETA, S. & YAMAMOTO, N. (1987). Inhibitory effect of glycyrrhizin on the *in vitro* infectivity and cytopathic activity of the human immunodeficiency virus [HIV (HTLV-III/LAV)]. Antiviral Research, **7**, 127-137.

ITO, M., SATO, A., HIRABAYASHI, K., TANABE, F., SHIGETA, S., BABA, M., DE CLERCQ, E., NAKASHIMA, H. & YAMAMOTO, N. (1988). Mechanism of inhibitory effect of glycyrrhizin on replication of human deficiency virus (HIV). Antiviral Research, **10**, 289-298.

- JARRETT, W.F.H., MARTIN, W.B., CRIGHTON, G.W., DALTON, R.G. & STEWART, M.F. (1964). Leukemia in the cat: Transmission experiments with leukemia (lymphosarcoma). Nature, **202**, 566-567.
- JARRETT, O., LAIRD, H.M. & HAY, D. (1973). Determinants of the host range of feline leukemia viruses. Journal of General virology, **20**, 169-175.
- JARRETT, O., HARDY, W.D.Jr., GOLDBERGER, M.C. & HAY, D. (1978). The frequency of occurrence of feline leukemia virus subgroups in cats. International Journal of Cancer, **21**, 334-337.
- JOHNSTON, B., LINDUP, W.E., SHILLINGFORD, J.S., SMITH, M. & PARKE, D.V. (1974). The pharmacological biochemistry of carbonoxolone. Its effect on gastric mucus. In 4th symposium on carbonoxolone (Avery Jones F, Parke DV, eds) pp 3-24. Butterworths, London.
- JOHNSON, V.A., WALKER, B.D., BARLOW, M.A., PARADIS, T.J. & CHOU, T.C. (1989). Synergistic inhibition of human immunodeficiency virus type 1 and type 2 replication in vitro by castanospermine and 3'-azido-3'-deoxythymidine. Antimicrobial Agents and Chemotherapy, **33**, 53-57.
- JOHNSON, V.A., MERRILL, D.P., CHOU, T.-C. & HIRSCH, M.S. (1992). Human immunodeficiency virus type 1 (HIV-1) inhibitory interactions between protease inhibitor RO 31-8959 and Zidovudine, 2', 3'-dideoxycytidine, or recombinant interferon- α against Zidovudine-sensitive or resistant HIV-1 in vitro. Journal of Infectious Diseases, **166**, 1143-1146.
- KAKUMA, S. (1993). Immunologic mechanism of glycyrrhizin. As reported in the satellite symposium on therapeutic approach to chronic active liver disease. The 1993 international symposium on viral hepatitis and liver disease Tokyo.
- KAN, N.C., FRANCHINI, G., WONG-STAAL, F., DUBOIS, G.C., ROBEY, W.C., LAUTENBERGER, J.A. & PAPAS, T.S. (1986). Identification of the HTLVIII/LAV sor gene product, and detection of antibodies in human sera. Science, **231**, 1553-1555.
- KATO, I., YASUNAGA, T., IKAWA, Y. & YOSHINAKA, Y. (1987). Inhibition of retroviral protease activity by an aspartyl proteinase inhibitor. Nature, **329**, 654-656.
- KAWAGUCHI, Y., MIYAZAWA, T., HORIMOTO, T., ITAGAKI S.I., FUKASAWA, M., TAKAHASHI, E. & MIKAMI, T. (1991). Activation of feline immunodeficiency virus long terminal repeats by feline herpesvirus type 1. Virology, **184**, 449-454.

KAWAGUCHI, Y., NORIMINE, J., MIYAZAWA, T., KAI, C. & MIKAMI, T. (1992). Sequence within the feline immunodeficiency virus long terminal repeat that regulate gene expression and respond to activation by feline herpesvirus type 1. Virology, **190**, 465-468.

KELLAM, P., BOUCHER, C.A.B. & LARDER, B.A. (1992). Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. Proceedings of the National Academy of Sciences USA, **89**, 1934-1938.

KHAN, A.S. & STEPHENSON, J.R. (1977). Feline leukemia virus: Biochemical and immunological characterisation of gag gene-coded structural proteins. Journal of Virology, **23**, 599-607.

KIM, S., BYRN, R., GROOPMAN, J. & BALTIMORE, D. (1989). Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. Journal of Virology, **63**, 3708-3713.

KIMURA, M., WATANABE, H. & ABO, T. (1992). Selective activation of extrathymic T cells in the liver by glycyrrhizin. Biotherapy, **5**, 167-176.

KIYOMASU, T., MIYAZAWA, T., FURUYA, T., SHIBATA, R., SAKAI, H., SAKURAGI, J.I., FUKASAWA, M., MAKI, N., HASEGAWA, A., MIKAMI, M. & ADACHI, A. (1991). Identification of feline immunodeficiency virus rev gene activity. Journal of Virology, **65**, 4539-4542.

KLIMKAIT, T., STREBEL, K., HOGGAN, M.D., MARTIN, M.A. & ORENSTEIN, J.M. (1990). The human immunodeficiency virus type 1-specific protein Vpu is required for efficient maturation and release. Journal of Virology, **64**, 621-629.

KOHL, N.E., EMINI, E.A., SCHLEF, W.A., DAVIS, L.J. & HEIMBACH, J.C. (1988). Active human immunodeficiency virus protease is required for viral infectivity. Proceedings of the National Academy of Sciences USA, **85**, 4686-4690.

KOHLSTEAD, L.A., WANG, J., FRIEDMAN, J.M., RICE, P.A. & STEITZ, T.A. (1992). Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science, **256**, 1783-1790.

KOSHY, R., GALLO, R.C. & WONG-STAAAL, F. (1980). Characterisation of the endogenous feline leukemia virus-related DNA sequences in cats and attempts to identify exogenous viral sequences in tissues of virus-negative leukemic animals. Virology, **103**, 434-445.

LITTLER, E., ZEUTHEN, J., MCBRIDE, A.A., TROST-SORENSEN, E., POWELL, K.L., WALSH-ARRAND, J.E. & ARRAND, J.R. (1985). Identification of an Epstein-Barr virus encoded thymidine kinase. EMBO Journal, **5**, 1939-1946.

KOWALSKI, M., POTZ, J., BASINPOUR, L., DORFMAN, T., GOH, W.C., TERWILLIGER, E., DAYTON, A., ROSEN, C., HASELTINE, W. & SODROSKI, J. (1987). Functional regions of the envelope glycoprotein of the human immunodeficiency virus type 1. Science, **237**, 1351-1355.

KTISTAKIS, N.T., ROTH, M.G. & BLOOM, G.S. (1991) Ptk₁ cells contain a non-diffusable, dominant factor that makes the Golgi apparatus resistant to Brefeldin A. Journal of Cell Biology, **113**, 1009-1023.

LARDER, B.A., DARBY, G., & RICHMAN, D.D. (1989). HIV with reduced sensitivity to Zidovudine (AZT) isolated during prolonged therapy. Science, **243**, 1731-1734.

LARDER, B.A. & KEMP, S.D. (1989a). Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science, **246**, 1155-1158.

LARDER, B.A. (1992). 3'-Azido-3'-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. Antimicrobial Agents and Chemotherapy, **36**, 2664-2669.

LASPIA, M.F., RICE, A.P. & MATHEWS, M.B. (1989). HIV-1 TAT protein increases the transcriptional initiation and stabilises elongation. Cell, **59**, 283-292.

LEE, T.H., COLIGAN, J.E., ALLAN, J.S., McLANE, M.F., GROOPMAN, J.E. & ESSEX, M. (1986). A new HTLV-III/LAV protein encoded by a gene found in a cytopathic retrovirus. Science, **231**, 1546-1549.

LERNER, D.L., WAGAMAN, P.C., PHILLIPS, T.R., PROSPERO-GARCIA, O., HENRIKSEN, S.J., FOX, H., BLOOM, F.E. & ELDER, J.H. (1995). Increased mutation frequency of feline immunodeficiency virus lacking functional deoxyuridine-triphosphate. Proceedings of the national of Academy of Sciences, **92**, 7480-7484.

LEVY, L.S. LOBELLE-RICH, P.A., OVERBAUGH, J., ABKOWITZ, J.L., FULTON, R. & ROY-BURMAN, P. (1993). Coincident involvement of flv-2, c-myc, and novel env genes in natural and experimental lymphosarcomas induced by feline leukemia virus. Virology, **196**, 892-895.

LIANG, X., BABIUK, L.A., VAN DRUNEN LITTEL-VAN DEN HURK, S., FITZPATRICK, D.R. & ZAMB, T.J. (1991). Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. Journal of Virology, **65**, 1124-1132.

LITTLER, E., ZEUTHEN, J., MCBRIDE, A.A., TROST-SORENSEN, E., POWEL, K.L., WALSH-ARRAND, J.E. & ARRAND, J.R. (1986). Identification of an Epstein-Barr virus encoded thymidine kinase. EMBO Journal, **5**, 1959-1966.

- LITTLER, E., STUART, A.D. & CHEE, M.S.** (1992). Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analog ganciclovir. Nature (London), **358**, 160-162.
- LOMBARDI, S., GARZELLI, C., La ROSA, C., ZACCARO, L., SPECTER, S., MALVALDI, G., TOZZINI, F., ESPOSITO, F. & BENDINELLI, M.** (1993). Identification of a linear neutralization site within the third variable region of feline immunodeficiency virus envelope. Journal of Virology, **67**, 4742-4749.
- LOMBARDI, S., MASSI, C., TOZZINI, F., ZACCARO, L., BAZZICHI, A., BANDECCHI, P., La ROSA, C., BENDINELLI, M. & GARZELLI, C.** (1995). Epitope mapping of the V3 domain of feline immunodeficiency virus envelope glycoprotein by monoclonal antibodies. Journal of General Virology, **76**, 1893-1899.
- LUCIW, P.A., CHENG-MAYER, C. & LEVY, J.A.** (1987). Mutational analysis of the human immunodeficiency virus: the ORF B region down-regulates virus replication. Proceedings of the national Academy of Sciences, **84**, 1434-1438.
- MAKI, N., MIYAZAWA, T., FUKASAWA, A., HASEGAWA, A., HAYAMI, M., MIKI, K. & MIKAMI, T.** (1992). Molecular characterisation and heterogeneity of feline immunodeficiency virus isolates. Archives of Virology, **123**, 29-45.
- MAHER, HIL.J., WOLD, B. & DERVAN, P.B.** (1989). Inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation. Science, **245**, 725-726.
- MALIM, M.H., HABER, J., FENN, C. & CULLEN, B.R.** (1988). Immunodeficiency virus Rev trans-activator modulates expression of the viral regulatory gene. Nature, **335**, 181-183.
- MALIM, M.H., BOHNLEIN, S., HAUBER, J. & CULLEN, B.R.** (1989). Functional dissection of the HIV-1 Rev trans-activator, derivation of a trans-dominant repressor of Rev function. Cell, **58**, 204-214.
- MAR, E.-C., CHIOU, J.-F., CHENG, Y.-C. & HUANG, E.-SH.** (1985). Inhibition of cellular DNA polymerase α and human cytomegalovirus-induced DNA polymerase by the triphosphates of 9-(2-Hydroxyethoxymethyl) Guanine and 9-(1,3-Dihydroxy-2-propoxymethyl)Guanine. Journal of Virology, **53**, 776-780.
- MARCINIAK, R.A. & SHARP, P.A.** (1991). HIV-Tat protein promotes formation of more progressive elongation complexes. EMBO Journal, **10**, 4189-4196.
- MARSH, M., & HELENIUS, A.** (1989). Virus entry into animal cells. Advances in Virus Research, **36**, 107-151.

MARSDEN, H.S., CROMBIE, I.K. & SUBAK-SHARPE, J.H. (1976). Control of protein synthesis in herpesvirus-infected cells analysis of the polypeptides induced by wild type and sixteen temperature-sensitive mutants of HSV strain 17. Journal of general Virology, **31**, 347-372.

MARSDEN, H.S., MURPHY, M., McVEY, G.L., MACEACHRAN, K.A., OWSIANKA, A.M. & STOW, N.D. (1994). Role of the carboxy terminus of herpes simplex virus type 1 DNA polymerase in its interaction with UL42. Journal of General Virology, **75**, 3127-3135.

MARSHALL, R.L., RODRIGUEZ, L.L. & LETCHWORTH III. (1986). Characterization of envelope proteins of infectious bovine rhinotracheitis virus (Bovine herpesvirus 1) by biochemical and immunological methods. Journal of Virology, **57**, 745-753.

MARTIN, K. & HELENIUS, A. (1991). Nuclear transport of influenza virus ribonucleoprotein: the viral matrix protein (M1) promotes export and inhibits import. Cell, **67**, 117-130.

MARTIN, K. & HELENIUS, A. (1991a). Transport of incoming influenza virus nucleocapsids into the nucleus. Journal of Virology, **65**, 232-244.

MATTEUCCI, D., BALDINOTTI, F., MAZZETTI, P., PISTELLO, M., BANDECCHI, P., GHILARDUCCI, R., POLI, A., TOZZINI, F. & BENDINELLI, M. (1993). Detection of feline immunodeficiency virus in saliva and plasma by cultivation and polymerase chain reaction. Journal of Clinical Microbiology, **31**, 494-501.

MAYFIELD, J.E., GOOD, P.J., VANOORT, H.J., CAMPBELL, A.R. & REED, D.E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesvirus 1 (cooper strain). Journal of Virology, **47**, 259-264.

McCLURE, M.O., MASH, M. & WEISS, R.A. (1988). Human immunodeficiency virus infection of CD4 bearing cells occurs by a pH-independent mechanism. EMBO Journal, **7**, 513-518.

McCLURE, M.O., SOMMERFELT, M.A., MARSH, M. & WEISS, R.A. (1990). The pH independence of mammalian retrovirus infection. Journal of General Virology, **71**, 767-773.

MERLUZZI, V.J., HARGRAVE, K.D., LAMBADIA, M., GROZINGER, K., SKOOG, M., WU, J.C., SHIH, C.-K., ECKNER, K., HATTOX, S., ADAM, J., ROSETHAL, A.S., FAANES, R., ECKNER, R.J., KOUP, R.A. & SULLIVAN, J.L. Science, **250**, 1411-1413.

- MERUELO, D., GAD, L. & LAVIE, D. (1988). Therapeutic agents with dramatic antiretroviral activity and little toxicity at effective doses: Aromatic polycyclic dions hypericin and psuedohypericin. Proceedings of the National Academy of Sciences USA, **85**, 5230-5234.
- MELLORS, J.W., BAZMI, H.Z., SCHINAZI, R.F., ROY, B.M., HSIU, Y., ARNOLD, E., WEIR, J. & MAYERS, D. (1995). Novel mutations in reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. Antimicrobial Agents and Chemotherapy, **39**, 1087-1092.
- MIDDELTON, J.P., KHAN, W.A., COLLINSWORTH, G., HANNUN, Y.A. & MEDFORD, R.M. (1993). Heterogeneity of protein kinase C-mediated rapid regulation of Na⁺/K⁺-ATPase in kidney epithelial cells. Biophysical ACTA, **103**, 15958-15967.
- MILLER, M., SCHNIDER, J., SATHYANARAYANA, B.K., TOTH, M.V., MARSHALL, G.R., CLAWSON, L., SLEK, L., KENT, S.B.H. & WLODAWER, A. (1989). Structure of complex of synthetic HIV-1 protease with a substrate base inhibitor at 2.3Å resolution. Science, **246**, 1149-1152.
- MILLER, M., JASKOLSKI, M., RAO, J.K.M., LEIS, J. & WLODAWER, A. (1989). Crystal structure of a retroviral protease proves relationship to aspartic protease family. Nature, **337**, 576-579.
- MISRA, V., BLUMENTHAL, R.M. & BABIUK, L.A. (1981). Proteins specified by bovine herpesvirus 1 (infectious bovine rhinotracheitis virus). Journal of Virology, **40**, 367-378.
- MITSUYA, H. & BRODER, S. (1986). Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2', 3'-dideoxynucleosides. Proceedings of the National Academy of Sciences USA, **83**, 1911-1915.
- MITSUYA, H. & BRODER, S. (1987). Strategies for antiviral therapy in AIDS. Nature, **325**, 773-778.
- MIYAZAWA, T., FUKASAWA, M., HASEGAWA, A., MAKI, N., IKUTA, K., TAKAHASHI, E., HAYAMI, M. & MIKAMI, T. (1991). Molecular cloning of a novel isolate of feline immunodeficiency virus biologically and genetically different from the original U.S. isolates. Journal of Virology, **65**, 1572-1577.
- MIYAZAWA, T., KAWAGUCHI, Y., KOHMOTO, M., SAKURAGI, J.I., ADACHI, A., FUKASAWA, M. & MIKAMI, T. (1992). Production of feline immunodeficiency virus in feline and non-feline non-lymphoid cell lines by transfection of an infectious molecular clone. Journal of General Virology, **73**, 1543-1546.

- MIYAZAWA, T., KOHOMOTO, M., KAWAGUCHI, Y., TOMONAGA, K., TOYOSAKI, T., IKUTA, K., ADACHI, A. & MIKAMI, T. (1993). The AP-1 binding site in the feline immunodeficiency virus long terminal repeats is not required for virus replication in feline T lymphocytes. Journal of General Virology, **74**, 1573-1580.
- MIYAZAWA, T., TOMONAGA, K., KAWAGUCHI, Y. & MIKAMI, T. (1994). The genome of feline immunodeficiency virus. Archives of Virology, **134**, 221-234.
- MOLHUYSEN, J.A., GERBRANDY, J., DEVRIES, L.A., DEJONG, J.C., LESNTRA, L.B., TURNER, K.P. & BORST, J.C. (1990). A liquorice extract with deoxycortone-like action. Lancet, **2**, 381-386.
- MONECH, T.R., WHALEY, K.J., MANDRELL, T.D., BISHOP, B.D., WITT, C.J. & CONE, R.A. (1993). The cat/feline immunodeficiency virus model for transmucosal transmission of AIDS: Nonoxynol-9 contraceptive jelly blocks transmission by an infected cell inoculum. AIDS, **7**, 797-802.
- MORI, K., SAKAI, H., SUZUKI, S., SUGAL, K., AKUTSU, Y., ISHIKAWA, M., SEINO, Y., ISIDA, N., UCHIDA, T., KAIYONE, S., ENDO, Y. & MIURA, A. (1989). Effect of glycyrrhizin (SNMC: stronger neo-minophagen C) in haemophilia patients with HIV infection. Tohoku Journal of Experimental Medicine, **158**: 25-35.
- MORI, K., SAKAI, H., SUZUKI, S., AKUTSU, Y., ISHIKAWA, M., IMAIZUMI, M., TADA, K., AIHARA, M., SAWADA, Y., YOKOYAMA, M., SATO, Y., ENDO, Y., SUZUKI, Z., SATO, S., SASAKI, H., YOKOYAMA, S., HAYASHI, T., UCHIDA, T., HIWATASHI, K., ISHIDA, N., FUJIMAKI, M. & YAMADA, K. (1990). Effects of glycyrrhizin (SNMC: stronger neo-minophagen C) in haemophilia patients with HIV-1 infection. Tohoku Journal of Experimental Medicine, **162**, 183-193.
- MORIKAWA, S., BOOTH, T.F. & BISHOP, D.H.L. (1991). Analysis of the requirements for the synthesis of virus-like particles by feline immunodeficiency virus gag using baculovirus vector. Virology, **183**, 288-297.
- MORIKAWA, S. & BISHOP, D.H.L. (1992). Identification and analysis of the gag-pol ribosomal frameshift site of feline immunodeficiency virus. Virology, **186**: 389-397.
- MUESING, M.A., SMITH, D.H. & CAPON, D.J. (1987). Regulation of mRNA accumulation by a human immunodeficiency virus trans-activator protein. Cell, **48**, 691-701.
- NAESENS, L., NEYTS, J., BALZARINI, J., HOLY, A., ROSENBERG, I. & De CLERCQ, E. (1993). Efficacy of oral 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) in the treatment of retrovirus and cytomegalovirus infections in mice. Journal of Medical Virology, **39**, 167-172.

- NEIL, J.C., ONIONS, D.E. & JARRETT, O. (1980). Polypeptides of feline leukemia virus: Identification of p15 (E) and p12 (E). Journal of General Virology, **50**, 455-460.
- NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. Science, **233**, 305-312.
- NISHIZUKA, Y. (1988). The molecular heterogeneity of protein kinase C and its implication for cellular regulation. Nature, **334**, 661-665.
- NUMAZAKI, K., UMETSUE, M. & CHIBA, S. (1994). Effect of glycyrrhizin in children with liver dysfunction associated with cytomegalovirus infection. Tohoku Journal of Experimental Medicine, **172**, 147-153.
- OHTSUKI, K. & NAKAO, I. (1988). Inhibitory effect of glycyrrhizin on polypeptide phosphorylation by polypeptide-dependent protein kinase (kinase P) *In vitro*. Biochemical and Biophysical Research Communications, **157**, 597-604.
- OLMSTED, R.A., HIRSCH, V.M., PURCELL, R.H. & JOHNSON, P.R. (1989). Nucleotide sequence analysis of feline immunodeficiency virus: Genome organization and relationship to other lentiviruses. Proceedings of the National Academy of Sciences USA, **86**, 8088-8092.
- OLMSTED, R.A., LANGLEY, R., ROELKE, M.E., GOEKEN, R.M., ADGER-JOHNSON, D., GOFF, J.P., ALBERT, J.P., PACKER, C., LAURENSEN, M.K., CARO, T.M., SCHEEPERS, L., WILDT, D.E., BUSH, M., MARTENSON, J.S. & O'BRIEN, S.J. (1992). Worldwide prevalence of lentivirus infection in wild feline species: epidemiologic and phylogenetic aspects. Journal of Virology, **66**, 6008-6018.
- ONEIL, L.L., BURKHARD, M.J., DIEHL, L.J. & HOOVER, E.A. (1995). Vertical transmission of feline immunodeficiency virus. AIDS Research and Human Retroviruses, **11**, 171-182.
- ORENTAS, R.J. & HILDRETH, J.E.K. (1993). Association of host cell surface adhesion receptors and other membrane proteins with HIV and SIV. AIDS Research and Human Retroviruses, **9**, 1157-1165.
- OSTRANDER, M. & CHENG, Y-C. (1980). Properties of herpes simplex type 1 and type 2 DNA polymerase. Biochemistry and Biophysical ACTA, **609**, 232-245.
- OVERBAUGH, J., RIEDEL, N., HOOVER, E.A. & MULLINS, J.I. (1988). Transduction of endogenous envelope genes by feline leukemia virus *in vitro*. Nature, **332**, 731-734.
- OVERBAUGH, J., DONAHUE, P.R., QUACKENBUSH, S.L., HOOVER, E.A. & MILLINS, J.I. (1988). Molecular cloning of a feline leukemia virus that induces fatal immunodeficiency disease in cats. Science, **239**, 906-910.

OXFORD, J.S., LOGAN, I.S. & POTTER, C.W. (1970). Passage of influenza strains in the presence of amino adamantane. Nature, **226**, 82-83.

PALMER, S. & COX, S. (1994). Intracellular metabolism of 3'-azido-3'-deoxythymidine and 2', 3' -dideoxyinosine in combination in the absence and presence of ribavirin. Antiviral Chemistry and Chemotherapy, **5**, 403-409.

PALMER, S. & COX, S. (1995). Intracellular activation and cytotoxicity of three different combinations of 3'-Azido-3'-deoxythymidine and 2', 3' -Dideoxyinosine. AIDS Research and Human Retroviruses, **11**, 1227-1233.

PANCINO, G., FOSSATI, I., CHAPPEY, C., CASTELOT, S., HURTEL, B., MORILLON, A., KLATZMANN, D. & SONIGO, P. (1993). Structure and variations of feline immunodeficiency virus envelope glycoproteins. Virology, **192**, 659-662.

PANCINO, G., CHAPPEY, C., SAURIN, W. & SONIGO, P. (1993a.) B epitopes and selection pressures in feline immunodeficiency virus envelope glycoproteins. Journal of Virology, **67**, 664-672.

PARTRIDGE, M. & POSWILLO, D.E. (1984). Topical carbenoxolone sodium in the management of herpes simplex infection. British Journal of Oral and Maxillofacial surgery, **22**, 138-145.

PAUWELS, R., ANDRIES, K., DESMYTER, J., SCHOLS, D., KUKLA, M.J., BRESLIN, H.J., RAEYMAECKERS, A., GELDER, J.V., WOESTENBORGH, R., HEYKANTS, J., SCHELLEKENS, K., JANSSEN, M.A.C., DECLERCQ, E. & JANSSEN, P.A.J. (1990). Potent and selective inhibition of HIV-1 replication by a novel series of TIBO derivatives. Nature, **343**, 471-474.

PEDERSON, N.C., HO, E.W., BROWN, M.L. & YAMAMOTO, J.K. (1987). Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. Science, **235**, 790-793.

PHILLIPS, T.R., TALBOTT, R.L., LAMONT, C., MUIR, S., LOVELACE, K. & ELDER, J.H. (1990). Comparison of two host cell range variants of feline immunodeficiency virus. Journal of Virology, **64**, 4605-4613.

PHILLIPS, T.R., LAMONT, C., KONINGS, D.A.M., SHACKLETT, B.L., HAMSON, C.A., LUCIW, P.A. & ELDER, J.H. (1992). Identification of the Rev transactivation and Rev-responsive element of feline immunodeficiency virus. Journal of Virology, **66**, 5464-5471.

PIEKEN, W.A., OLSEN, D.B., BENSELER, F., AURUP, H. & ECKSTEIN, F. (1991). Kinetic characterisation of ribonuclease-resistant 2'-modified hammerhead ribozymes. Science, **253**, 314-317.

POLIS, M.A., SPOONER, K.M., BAIRD, B.F., MANISCHEWITZ, J.F., JAFFE, H.S., FISHER, P.E., FALLOON, J., DAVEY, R.T., KOVACS, J.A., WALKER, R.E., WHITCUP, S.M., NUSSENBLATT, R.B., LANE, H.C. & MASUR, H. (1995). Anticytomegalovirus activity and safety of Cidofovir in patients with human immunodeficiency virus infection and cytomegalovirus viruria. Antimicrobial Agents and Chemotherapy, **39**, 882-886.

POMPEI, R., FLORE, O., MARCCIALIS, M.A., PANI, A. & LODDO, B. (1979). Glycyrrhizic acid inhibits virus growth and inactivates virus particles. Nature, London, **281**, 689-690.

POMPEI, R., FLORE, O., PANI, A., MARCCIALIS, M.A. & MARCNGIU, M. E. (1979). On the antiviral action of glycyrrhizic acid II- Non involvement of cell membrane transport of ions and metabolites in the antiviral effect. Rivista Di Farmacologia e Terapia, **X**, 335-359.

POMPEI, R., PANI, A., FLORE, O., MARCCIALIS, M.A. & LODDO, B. (1980). Antiviral activity of glycyrrhizic acid. Experientia, **36**, 304.

POMPEI, R. (1981). Impaired recovery of infectious herpes simplex virus from glycyrrhizic acid treated cells. Rivista Di Farmacologia e Terapia, **XII**, 43-46.

POMPEI, R., PAGHI, L., INGIANNI, A. & UCCHEDDU, P. (1983). Glycyrrhizic acid inhibits influenza virus growth in embryonated eggs. Microbiologica, **6**, 247-250.

POSWILLO, D.E. & ROBERTS, G.J. (1981). Topical carbenoxolone for orofacial herpes simplex infections. Lancet, **2**, 143-144.

PRIZER, L.I., COHN, G.H. & EISENBERG, R.J. (1980). Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. Journal of Virology, **34**, 142-153.

PRUSOFF, W.H., MANCINI, W.R., LIN, T-S., LEE, J.J., SIEGEL, S.A. & OTTO, M.J. (1984). Physical and biological consequences of incorporation of antiviral agents into virus DNA. Antiviral Research, **4**, 303-315.

PRUSOFF, W.H. & LIN, T-S. (1988). Experimental aspects of antiviral pharmacology, in : "ANTIVIRAL DRUG DEVELOPMENT " pp173-202. Edited by E. De CLERCQ and R.T WALKER

PU, R., OKADA, S., LITTLE, E.R., XU, B., STOFFS, W.V. & YAMAMOTO, J.K. (1995). Protection of neonatal kittens against feline immunodeficiency virus infection with passive maternal antiviral antibodies. AIDS, **9**, 235-242.

PUE, M.A. & BENET, L.Z. (1993). Pharmacokinetics of famciclovir in man. Antiviral Chemistry and Chemotherapy, **4**, 47-55.

REARDON, P.M. & AUDUS, K.L. (1993). Ammonium glycyrrhizinate (AMGZ) effects on membrane integrity. International Journal of Pharmaceutics, **94**, 161-170.

REID, R., MAR, E-C., HUANG, E-S. & TOPAL, M.D. (1988). Insertion and extension of acyclic, dideoxy, and ara nucleotides by herpesviridae, human α and human β polymerases. Journal of Biological Chemistry, **263**, 3898-3904.

RHO, H.M. & GALLO, R.C. (1979). Characterisation of reverse transcriptase from feline leukemia virus by radioimmunoassay. Virology, **99**, 192-196.

RICHMAN, D., ROSENTHAL, A.S., SKOOG, M., ECKNER, R.J., CHOU, T.-C., BABO, J.P. & MERLUZZI, V.J. (1991). BI-RG-587 is active against zidovudine-resistant human immunodeficiency virus type 1 and synergistic with zidovudine. Antimicrobial Agents and Chemotherapy, **35**, 305-308.

RICHMAN, D.D., SHIH, C.-K., LOWY, I., ROSE, J., PRODANOVICH, P., GOFF, S. & GRIFFIN, J. (1991a). HIV-1 mutants resistant to nucleoside inhibitors of reverse transcriptase arise in tissue culture. Proceedings of the national Academy of Sciences USA, **88**, 11241-11245.

RICHMAN, D.D., HAVLIR, D., CORBEIL, J., LOONEY, D., IGNACIO, C., SPECTOR, S.A., SULLIVAN, J., CHEESMAN, S., BARRINGER, K., PAULETTI, D., SHIH, C.-K., MAYER, M. & GRIFFIN, J. (1994). Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. Journal of Virology, **68**, 1660-1666.

RIGBY, M.A., HOLMS, E.C., PISTELLO, M., MACKAY, A., LEIGH BROWN, A.J. & NEIL, J.C. (1993). Evolution of structural proteins of feline immunodeficiency virus: molecular epidemiology and evidence of selection for change. Journal of General Virology, **74**, 425-436.

ROBERTS, N.A., MARTIN, J.A., KINCHINGTON, D., BROADHURST, A.V., CRAIG, J.C., DUNCAN, I.B., GALPIN, S.A., HANDA, B.K., KAY, J., KROHN, A., LAMBERT, R.W., MERRETT, J.H., MILLS, J.S., PARKES, K.E.B., REDSHAW, S., RITCHIE, A.J., TAYKOR, D.L., THOMAS, G.J. & MACHIN, P.J. (1990). Rational design of peptide-based HIV proteinase inhibitors. Science, **241**, 358-361.

- ROBERTS, J.D., BEBENEK, K. & KUNEL, T.A.** (1988). The accuracy of reverse transcriptase from HIV-1. Science, **242**, 1171-1173.
- RHON, J.L. & OVERBAUGH, J.** (1995). In vivo selection of long terminal repeat alterations in feline leukemia virus-induced thymic lymphomas. Virology, **206**, 661-665.
- ROSEN, C.A., TERWILLIGER, E., DAYTON, A., SODROSKI, J.G. & HASELTINE, W.A.** (1988). Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus. Proceedings of the National Academy of Sciences USA, **85**, 2071-2075.
- SARMA, P.S. & LOG, T.** (1973). Subgroup classification of feline leukemia and sarcoma viruses by viral interference and neutralization tests. Virology, **54**, 160-169.
- SARVER, N., CANTIN, E.M., CHANG, P.S., ZAIA, J.A., LADNE, P.A., STEPHENS, D.A. & ROSSI, J.J.** (1989). Robozymes as potential anti-HIV therapeutic agents. Science, **247**, 1222-1225.
- SATO, A., IGARASHI, H., ADACHI, A. & HAYAMI, M.** (1990). Identification and localisation of vpr gene product of human immunodeficiency virus type 1. Virus Genes, **4**, 303-312.
- SAUL, R., GHIDONI, J.J., MOLYNEX, R.J. & ELBIEN, A.D.** (1985). Castanospermine inhibits glucosidase activities and alters glycogen distribution in animals. Proceedings of the National Academy of Sciences USA, **82**, 93-97.
- ASCHLESINGER, M.J., ASHBURNER, M. & TISSIERES, A.** (1982). Heat Shock from bacteria to man. COLD SPRING HARBOR LABORATORY, COLD SPRING HARBOR, NEW YORK.
- SCHMID, S. L., FUCHS, R., MALE, P. & MELLMAN, I.** (1988). Two distinct subpopulation of endosomes involved in membrane recycling and transport to lysosomes. Cell, **52**, 73-83.
- SHACKLETT, B. & LUCIW, P.A.** (1994). Analysis of the VIF gene of feline immunodeficiency virus. Virology, **204**, 860-867.
- SHEETS, R.L., PANDY, R., KLEMENT, V., GRANT, C.K. & ROY-BURMAN, P.** (1992). Biologically selected recombinants between Feline leukemia virus (FeLV) subgroup A and an endogenous FeLV element. Virology, **190**, 849-855.
- SHERIDAN, P.L., SHELINE, C.T., MILOCO, L.H., & JONES, K.A.** (1993). Tat and the HIV-1 promotor. a model for RNA-mediated regulation and transcription. Seminars in Virology, **4**, 69-80.

- SHIH, C.-K., ROSE, J.M., HANSEN, G.L, WU, J.C., BACOLLA, A. & GRIFFIN, J.A. (1991). Chimeric human immunodeficiency virus type 1/type2 reverse transcriptase display reversed sensitivity to nucleoside analog inhibitors. Proceedings of the national Academy of Sciences USA, **88**, 9878-9882.
- SIDEWELL, R.W., HUFFMAN, J.H., KHARKE, G.P., ALLEN, L.B., WITOWSKI, J.T. & ROBINS, R.K. (1972). Broad spectrum antiviral activity of viazole : 1-B-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide. Science, **177**, 705-706.
- SIEGEL, L.J., RATNER, L., JOSEPHS, S.F., DERSE, D., FEINBERG, M.B., REYES, G.R., O'BRIEN, S.J. & WONG-STAAAL, F. (1986). Trans-activation induced by human T-lymphotropic virus type III (HTLV-III) maps to viral sequence encoding 58 amino acids and lacks tissue specificity. Virology, **148**, 226-231.
- SIEKEVITZ, M., JOSEPHS, S.F., DUKOVICH, M., PEFFER, N., WONG-STAAAL, F. & GREENE, W.C. (1987). Activation of the HIV-1 LTR by T cell mitogen and the trans-activator protein of HTLV-1. Science, **238**, 1575-1578.
- SKALKA, A.M. (1989). Retroviral protease: first glimpses of the anatomy of a processing machine. Cell, **56**, 911-913.
- SMEE, D.F., MARTIN, J.C., VERHEYDEN, J.P. & MATTEWS, T.R. (1983). Anti-herpes virus activity of the cyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl) guanine. Antimicrobial Agents and Chemotherapy, **23**, 676-682.
- SNOEK, R., SAKUMA, T., De CLERCQ, E., ROSENBERG, I. & HOLY, A. (1988). (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine a potent and selective inhibitor of human cytomegalovirus replication. Antimicrobial Agents and Chemotherapy, **32**, 1839-1844.
- SODORA, D.L., SHAPER, E.G., KITCHELL, B.E., DOW, S.W., HOOVER, E.A. & MULLINS, J.I. (1994). Identification of three feline immunodeficiency virus (FIV) env gene subtypes and comparison of the FIV and human immunodeficiency virus type I evolutionary patterns. Journal of Virology, **68**, 2230-2238.
- SOE, L.H., DEVI, B.G., MULLINS, J.I. & ROY-BURMAN, P. (1983). Molecular cloning and characterisation of endogenous feline leukemia virus sequences from a cat genomic library. Journal of Virology, **46**, 829-840.
- SORGER, P.K. & PELHAM, H.R.B. (1987). The glucose-related protein grp94 is related to heat shock protein hsp 90. Journal of Molecular Biology, **194**, 341-344.
- SPARGER, E.E., SHACKLETT, B.L., RENSHAW-GEGG, L., BARRY, P.A., PEDERSON, N.C., ELDER, J.H. & LUCIW, P.A. (1992). Regulation of gene expression directed by the long terminal repeat of the feline immunodeficiency virus. Virology, **187**, 165-177.

ST. CLAIR, M.H., MARTIN, J.L., TUDOR-WILLIAMS, G., BACH, M.C., VAVRO, C.L., KING, D.M., KELLAM, P., KEMP, S.D. & LARDER, B.A. (1991). Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science, **253**, 1557-1559.

STANAT, S.C., REARDON, J.E., ERICE, A., JORDAN, M.C., DREW, W.L. & BRION, K.K. (1991). Ganciclovir-resistant cytomegalovirus clinical isolate: mode of resistance to ganciclovir. Antimicrobial Agents and Chemotherapy, **35**, 2191-2197.

STEINMAN, R., DOMBROWSKI, J., O'CONNOR, T., MONTELARO, R.C., TONELLI, Q., LAWRENCE, K., SEYMOUR, C., GOODNESS, J., PEDERSON, N.C. & ANDERSEN, R. (1990). Biochemical and immunological characterisation of the major structural proteins of feline immunodeficiency virus. Journal of General Virology, **71**, 701-706.

STEPHENS, E.B., MONCK, E.A. & BUTFTLOSKI, E.J. (1991). Processing of the glycoprotein of feline immunodeficiency virus: effect of inhibitors of glycosylation. Journal of Virology, **65**, 1114-1123.

STEVENSON, M., HAGGERTY, S., LAMONICA, C.A., MEIER, G.M., WELCH, S-K. & WASIAK, A.J. (1990). Integration is not necessary for expression of human immunodeficiency virus type 1 products. Journal of Virology, **64**, 2421-2425.

STEWART, M.A., WARNOCK, M., WHEELER, A., WILKIE, N., MULLINS, J.I., ONIONS, D.E. & NEIL, J.C. (1986). Nucleotide sequences of a feline leukemia virus subgroup A envelope gene and long terminal repeat and evidence for the recombinational origin of subgroup B viruses. Journal of Virology, **58**, 825-834.

STEWART, P.M., WALLACE, A.M., VALENTINO, R., BURT, D., SHACKELTON, C.H.L. & EDWARDS, C.R.W. (1987). Mineral corticoid activity of liquorice: 11-Beta-hydroxysteroid dehydrogenase deficiency comes of age. Lancet, 821-824.

STREBEL, K., DAUGHERTY, D., CLOUSE, K., COHEN, D., FOLKS, T. & MARTIN, M.A. (1987). The HIV' A' (sor) gene product is essential for virus infectivity. Nature, **328**, 728-730.

STREBEL, K., KLIMKAIT, T. & MARTIN, M.A. (1988). A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science, **241**, 1221-1223.

STREBEL, K., KLIMKAIT, T., MALDARELLI, F. & MARTIN, M.A. (1989). Molecular and biochemical analysis of human immunodeficiency virus type 1 Vpu protein. Journal of Virology, **63**, 3784-3791.

STREETER, D.G., WITOWSKI, J.T., KHARE, G.D., SIDEWELL, R.W., BAUER, R.J., ROBINS, R.K. & SIMON, L.N. (1973). Mechanism of action of 1-B-D-ribofuranosyl-1, 2,4-triazole-3-carboxamide. (Virazole), a new broad spectrum antiviral agent. Proceedings of the National Academy of Sciences USA, **70**, 1174-1178.

SUGRUE, R.J., BAHADUR, G., ZAMBON, M.C., HALL-SMITH, M., DOUGLAS, A.R. & HAY, A.J. (1990). Specific structural alteration of the influenza hemagglutinin by amantadine. EMBO Journal, **9**, 3469-3476.

SULLIVAN, V., TALAICO, C., STANAT, S.C., DAVIS, M., COEN, D.M. & BRION, K.K. (1992). A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature, **358**, 162-164.

SULLIVAN, V., BIRON, K.K., TALARICO, C., STANAT, S.C., DAVIS, M., POZZI, L.M. & COEN, D.M. (1993). A point mutation in the human cytomegalovirus DNA polymerase gene confers resistance to ganciclovir and phosphonylmethoxyalkyl derivatives. Antiviral Agents and Chemotherapy, **37**, 19-25.

SUZUKI, H., OHTA, Y., TAKINO, T., FUJISAWA, K. & HIRAYAMA, C. (1983). Effects of glycyrrhizin on biochemical tests in patients with chronic hepatitis. Asian Medical Journal, **26**, 423-438.

TACHEDIJIAN, G., HOY, J., MILLS, J. & BIRCH, C.J. (1994). Foscarnet therapy is not associated with the emergence of foscarnet-resistance human immunodeficiency virus type1 in an acquired immunodeficiency syndrome patient. Journal of Medical Virology, **42**, 207-211.

TAKAHARA, T., WATANABE, A., & SHIRAKI, K. (1994). Effect of glycyrrhizin on hepatitis B surface antigen: a biochemical and morphological study. Journal of Hepatology, **21**, 601-609.

TALBOTT, P.L., SPARGER, E.E., LOVELACE, K.M., FITCH, W.M., PEDERSON, N.C., LUCIW, P.A. & ELDER, J.H. (1989). Nucleotide sequence and genomic organization of feline immunodeficiency virus. Proceedings of the National Academy of Sciences USA, **86**, 5743-5747.

TEICH, N. (1982) Taxonomy of retroviruses RNA tumor viruses. Molecular biology of RNA tumor viruses (2nd edition). WEISS, R., TEICH, N., VARMUS, H. & COFFIN, J., (Eds). Cold Spring Harbour Press, New York.

TEICH, N. (1985). Taxonomy of retroviruses. RNA tumor viruses (2nd edition supplements and appendices). WEISS, R., TEICH, N., VARMUS, H., COFFIN, J. (Eds). Cold Spring Harbour Press, New York.

- TERWILLIGER, E.F., COHEN, E.A., LU, Y.C., SODROSKI, J.G. & HASELTINE, W.A.** (1989). Functional role of human immunodeficiency virus type 1 vpu. Proceedings of the National Academy of Sciences USA, **86**, 5136-5167.
- THREADGILL, D.S., STEAGALL, W.K., FLAHERTY, M.T., FULLER, F.J., PERRY, S.T., RUSHLOW, K.E., Le GRICE, S.F.J. & PAYNE, S.L.** (1993). Characterisation of equine infectious anemia virus dUTPase: growth properties of a dUTPase -deficient mutant. Journal of Virology, **67**, 2592-2600.
- TISDALE, M., MEYERS, R.E., MASCHERA, B., PARRY, N.R., OLIVER, N.M. & BLAIR, E.D.** (1995). Cross-resistance analysis of human immunodeficiency virus type 1 variants individually selected for resistance to five different protease inhibitors. Antimicrobial Agents and chemotherapy, **39**, 1704-1710.
- TOKUNAGA, K., NISHINO, Y., OIKAWA, H., ISHIHARA, C., MIKAMI, T. & IKUTA, K.** (1992). Altered cell tropism and cytopathicity of feline immunodeficiency viruses in two different feline CD4-positive, CD8-negative cell lines. Journal of Virology, **66**, 3893-3898.
- TOMKINS, A. M. & EDMONDS, C. J.** (1975). Electrical potential difference, sodium absorption and potassium secretion by the human rectum during carbenoxolone therapy. Gut, **16**, 277-284.
- TOMONAGA, K., NORIMINE, J., SHIN, Y.S., FUKASAWA, M., MIYAZAWA, T., ADACHI, A., TOYOSAKI, T., KAWAGUCHI, T., KAI, C. & MIKAMI, T.** (1992). Identification of a feline immunodeficiency gene which is essential for cell-free virus infectivity. Journal of Virology, **66**, 6181-6185.
- TOMONAGA, K., SHIN, Y.S., FUKASAWA, M., MIYAZAWA, T., ADACHI, A. & MIKAMI, T.** (1993). Feline immunodeficiency virus gene expression: analysis of the RNA splicing pattern and the monocistronic rev mRNA. Journal of General Virology, **74**, 2409-2417.
- TOMONAGA, K., MIYAZAWA, T., SAKURAGI, J., MORI, T., ADACHI, A. & MIKAMI, T.** (1993a). The feline immunodeficiency virus ORF-A gene facilitates efficient viral replication in established T cell lines and peripheral blood lymphocytes. Journal of Virology, **67**, 5889-5895.
- TORTEN, M., FRANCHINI, M., BARLOUGH, J.E. GEORGE, J.W. MOZES, E., LUTZ, H. & PEDERSEN, N.C.** (1991). Progressive immune dysfunction in cats experimentally infected with feline immunodeficiency virus. Journal of Virology, **65**, 2225-2230.

TOZZINI, F., MATTEUCCI, D., BANDECCHI, P., BADINOTTI, F., SIEBELINK, K.H.J., OSTERHAUS, A. & BENDINELLI, M. (1992). Neutralization antibodies in cats infected with feline immunodeficiency virus. Journal of Clinical Microbiology, **31**, 1626-1629.

TURANO, A., SCURA, G., CARUSO, A., BONFANTI, C., LUZATI, R., ET AL. (1989). Inhibitory effect of papaverine on HIV replication in vitro. AIDS Research and Human Retroviruses, **5**, 183-192.

TYMS, A.S., BERRLE, E.M., RYDER, T.A., WASH, R.J., HEGARTY, M.P. & TAYLOR, D.L. (1987). Castanospermine and other plant alkaloid inhibitors of glucosidase activity block the growth of HIV. Lancet, **11**, 1025-1026.

VAHLENKAMP, T.W., DE RONDE, A., BALZARINI, J., NAESENS, L., DE CLERCQ, E.D., VANEIJK, M.J.T., HORZINEK, M.C. & EGBERINK, H.F. (1995). (R)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine is a potent inhibitor of feline immunodeficiency virus infection. Antimicrobial Agents and Chemotherapy, **39**, 746-749.

VAN DRUNEN LITTEL-VAN DEN HURK, S. & BABIUK, L.A. (1986). Synthesis and processing of bovine herpesvirus 1 glycoproteins. Journal of Virology, **59**, 401-410.

VAN DRUNEN LITTEL-VAN DEN HURK, S., GARZON, S., VAN DEN HURK, J.V., BABIUK, L.A. & TUSSEN, P. (1995). The role of the major tegument protein VP8 of bovine herpesvirus-1 in infection and immunity. Virology, **206**, 413-425.

VELLA, S. (1994). Update on a proteinase inhibitor. AIDS, **8**, 25-29.

VINGE, P., FRELIN, C. & LAZUDUNSKI, M. (1982). The amiloride-sensitive Na^+/H^+ exchange system in skeletal muscle cells in culture. The Journal of Biological Chemistry, **257**, 9394-9400.

WALKER, B.D., KOWALSKI, M., GOTT, W.C., KOZARSKY, K., KRIEGER, M. & ROSEN, C. (1987). Inhibition of HIV syncytium formation and virus replication by castanospermine. Proceedings of the National Academy of Sciences USA, **84**, 8120-8124.

WALLBANK, A.M., MATTER, R.E. & KLINIKOWSKI, N.G. (1966). 1-adamantanamine hydrochloride inhibition of Rous and Esk sarcoma viruses in cell culture. Science, **152**, 1760-1761.

WEI, X., GHOSH, S.K., TAYLOR, M.E., JOHNSON, V.A., EMINI, E.A., DEUTSCH, P., LIFSON, J.D., BONHOEFFER, S., NOWAK, M.A., HAHN, B.H., SAAG, M.S. & SHAW, G.M. (1995). Viral dynamics in human immunodeficiency virus type 1 infection. Nature, **373**, 117-122.

WELCH, W.J., GARRELS, J.I., THOMAS, G.P., LIN, J.J.C. & FERAMISSO, J.R. (1983). Biochemical characterisation of the mammalian stress proteins and identification of 2 stress proteins as glucose and Ca^{2+} ionophore regulated proteins. Journal of Biological Chemistry, **258**, 7102-7111.

WHITELEY, R.M., SOONG, S-J, DOLIN, R., GALASSO, G., CH'EN, L.T. & ALFORD, C.A., THE COLLABORATIVE STUDY GROUP. (1977). Adenine arabinoside therapy of biopsy-proved herpes simplex encephalitis. New England Journal of Medicine, **297**, 289-294.

WILDHIRT, M.D. Experience in Germany with Glycyrrhizic acid for the treatment of chronic viral hepatitis. as reported in satellite symposium on therapeutic approach to the chronic active liver disease. The 1993 international symposium on viral hepatitis and liver disease Tokyo.

WILLETT, B.J., HOSIE, M.J., JARRETT, O. & NEIL, J.C. (1994). Identification of a putative cellular receptor for feline immunodeficiency virus as the feline homologue of CD9. Immunology, **81**, 228-233.

WIRTH, U.V., GUNKEL, K., ENGELS, M. & SCHWYZER, M. (1989). Spatial and temporal distribution of bovine herpesvirus 1 transcripts. Journal of Virology, **63**, 4882-4889.

WOLF, T.F., NARA, P.L. & GOUDSMIT, J. (1993). Genotypic and phenotypic variation of HIV: impact on AIDS pathogenic and vaccination. Chemical Immunology, **56**, 1-33.

WONG-STAAAL, F., CHANDA, P.K. & CHRAYEB, J. (1987). HIV: the eight gene. AIDS Research and Human Retroviruses, **3**, 33-39.

WRAY, S.K., GILBERT, B.E. & KNIGHT, V. (1985). Effect of ribavirin triphosphate on primer generation and elongation during influenza virus transcription *in vitro*. Antiviral Research, **5**, 39-48.

WRIGHT, C.W. (1995). Natural products in the fight against AIDS. The pharmaceutical Journal, **254**, 583-587.

YAMASHIRO, D. J., FLUSS, S.R. & MAXFIELD, F.R. (1983). Acidification of endocytic vesicles by an ATP-dependent proton pump. Journal of Cell Biology, **97**, 929-934.

YOSHIMURA, A., KURODA, K., KAWASAKI, S., MAEDA, T. & OHNISHI, S. (1982). Infectious cell entry of mechanism of influenza virus. Journal of Virology, **43**, 284-292.

ZABEDEE, S.L. & LAMB, R.A. (1988). Influenza A virus M2 protein monoclonal antibody restriction of virus growth and detection of M2 in virions. Journal of Virology, **62**, 2762-2772.

ZLYDNIKOV, D.M., KUBAR, O.I., KOVALEVA, T.P. & KAMFORIN, L.E. (1981). Study of rimantadine in the USSR: a review of the literature. Review of Infectious Diseases, **3**, 408-421.

